Identification of Cellular HIV Restriction Factors And Host-HIV Interactions

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Zusammenfassung

Das Humane Immundefizienz-Virus Typ 1 (HIV-1) ist das Pathogen, welches verantwortlich ist für das erworbene Immundefizienzsyndrom (AIDS). Die Verbreitung von HIV-1 hat sich zu einer Pandemie entwickelt, mit weltweit mehr als 35 Millionen infizierten Menschen und jährlich neuen Infektionen. Trotz der verfügbaren, effizienten antiretroviralen Therapie (ART) steigt das Erscheinen von Medikamentenresistenz mit der Zeit und der Zugang zu ART ist beschränkt in Entwicklungsländern. Aus diesem Grund ist es notwendig, neue Strategien zu entwickeln für das Blockieren der Virusreplikation und nach einer permanenten Heilung zu suchen.

Eines der Ziele dieser Doktorarbeit war die Identifizierung neuer zellulärer anti-HIV-1 Wirtsfaktoren, welche potenziell als neue therapeutische Ansätze benutzt werden können. Die meisten der bisher identifizierten Restriktionsfaktoren sind IFN-α-induzierbar und werden neutralisiert durch akzessorische HIV-1 Proteine. Wir suchten nach Restriktionsfaktoren in CD4+ T-Zellinien, in denen die HIV-1 Replikation stark inhibiert wurde durch IFN-α. Wir identifizierten 44 mögliche Kandidaten in den beiden Zellinien, die möglicherweise zwischen der reversen Transkription und Integration eingreifen.

Ein zweites Ziel dieser Doktorarbeit war es, ein verbessertes Wissen über den Beitrag der Hypermutationsaktivität des bekannten Restriktionsfaktors APOBEC3G (A3G) zur HIV-1 Evolution *in vivo* zu erlangen. A3G wurde hauptsächlich *in vitro* auf seine mutagene Kapazität untersucht. Zu diesem Zweck verwendeten wir die humanisierte Maus als Modell für die HIV-1 Infektion. In humanisierten Mäusen ist das Immunsystem defizient und wird rekonstituiert mit humanen hämatopoetischen Vorläuferzellen, die ein Immunsystem humaner Herkunft entwickeln, welches die HIV-1 Infektion in Mäusen erlaubt. Wir studierten Virusreplikation und Diversifizierung in Mäusen, die mit einer HIV-1 Mutante infiziert worden waren, welche unfähig ist, die Hypermutationsaktivität von A3G vollständig zu neutralisieren (Vif 45G). Wir beobachteten, dass die Infektion mit dieser Mutante nicht in einem erhöhten Erscheinen der Medikamentenresistenz resultierte, wie bisherig angenommen, sondern eher die virale Fitness von HIV-1 unter selektivem Druck erhöhte.

Summary

Human immunodeficiency virus type -1 (HIV-1) is the pathogen responsible for the acquired immunodeficiency syndrome (AIDS). HIV-1 is pandemic, affecting more than 35 million people worldwide and there are newly infected people every year. Despite the efficient anti-retroviral therapies (ART) available, the emergence of drug resistance increases overtime and the access to ART is very limited in developing countries. Therefore it is necessary to develop new strategies to block viral replication and to seek for a permanent cure.

Restriction factors are cellular proteins aiming at inhibiting pathogen invasion. One of the aims of this thesis was to identify new cellular host HIV-1 restriction factors, which potentially can be used as a new therapeutic approach. Key features or signatures of currently known restriction factors are i) IFN-α-inducible and ii) counteracted by HIV-1 accessory proteins. We screened for restriction factors in CD4+ T-cell lines in which HIV-1 replication was strongly inhibited by IFN-α. We identified 44 possible candidates that might act between viral reverse transcription and integration in two cell lines.

A second aim of this thesis was to evaluate or explore the contribution of the hypermutation activity of the known restriction factor APOBEC3G (A3G) to HIV-1 evolution *in vivo*. A3G has been mainly characterized *in vitro* for its mutagenic capacity. To this end, we used the humanized mouse as a model for HIV-1 infection. In humanized mice, the immune system is deficient and reconstituted with human hematopoietic precursor cells, which develop an immune system of human origin, allowing HIV- infection in mice. We studied viral replication and diversification in mice infected with a HIV-1 mutant which is unable to completely counteract A3G's hypermutation activity (Vif 45G). We observed that infection with this mutant did not result in an increased drug resistance appearance, as previously thought, but it rather increased the viral fitness of HIV-1 under selective pressure.

1. Introduction

The introduction of this thesis consists of three main parts. First, there is a short general overview about HIV-1. Second, a review focusing on the role of IFN-α in HIV-1 restriction, which will give the basis to the working hypothesis of this thesis. The working hypothesis is that there are several unknown IFN-α-dependent cellular factors that are able to restrict HIV-1 replication in CD4+ T cells. Third, there is a second review describing the use of the humanized mice for studying HIV-1, since the other part of my thesis work was focused on the study of the restriction factor APOBEC3G *in vivo*, using the humanized mouse model and also because I was involved in the experiments of my colleagues Marc Nischang and Renier Myburgh using the humanized mouse as a tool for novel anti-HIV therapies *in vivo* and for *in vivo* gene therapy against HIV-1, respectively (see manuscripts below).

1.1 The human immunodeficiency virus (HIV) type-1

HIV-1 results in a chronic infection and eventually in the acquired immunodeficiency syndrome (AIDS)[1]. HIV-1 belongs to the retroviridae family and in particular to the sub-family lentivirinae and the Lentivirus genus. Apart from HIV-1, there is a 2nd human pathogenic HIV strain, i.e., HIV-2 which, however, is much less pathogenic than HIV-1[2]. HIV-1 (subtypes M, N and O) is closely related to the simian immunodeficiency virus (SIV) of chimpanzees[3] or of gorillas (HIV-1 subtype P)[4]. HIV-2, is related to SIV from African green monkeys[2, 5]. HIV-1 is pandemic, while HIV-2 is limited to West Africa.

1.1.1 HIV epidemiology

According to the UNAIDS in 2013 35.3 million people on average were living with HIV and 2.3 million were newly infected in 2013. 1.6 million people died due to AIDS-related complications worldwide. Since the identification of AIDS in 1981, approximately 60 million people have been infected and 30 million already died (UNAIDS, Global report 2013).

Sub-Saharan Africa is the most affected region, with a prevalence of 4.9% accounting for 69% of total HIV infections. In South, East and South-East Asia, 5 million people are infected, representing 12% of all HIV infections. Finally, the Caribbean, Eastern Europe and Central Asia represent the third biggest reservoir of HIV-infected people, where 1% of the infected people live (UNAIDS, Global report 2012).

1.1.2 HIV Genome

All retroviruses are composed of two copies of positive single-stranded RNA (ssRNA) and their genomes contain i) the gag encoding for the matrix, capsid, nucleocapsid and p6, which make up the core of the virion , ii) the pol encoding for the HIV-1 enzymes, i.e., protease (PR), reverse transcriptase (RT) and integrase (IN) and iii) the env encoding the envelope protein which engages cell surface receptors for cellular entry[6-8]. The HIV genome with a length of ~9000 bp[9],which is a complex retrovirus as opposed for example to the moloney murine leukemia virus[10], encodes for (i) two regulatory proteins, Tat[11] and Rev[12], compulsory for transcription elongation and export of the unspliced RNA to the cytosol, respectively, and (ii) four accessory proteins including Vif, Vpr, Vpu and Nef for HIV-1 (reviewed in[13-15]) and Vif, Vpx, Vpr and Nef for HIV-2 (reviewed in[15]) (Fig. 1). The accessory proteins are essential for virulence *in vivo* (see below).

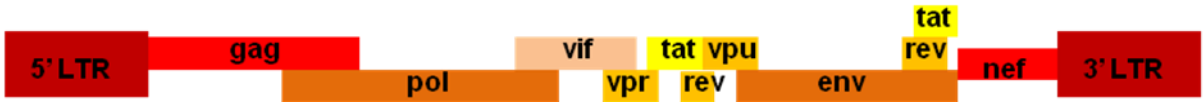

Fig. 1. Structure of the HIV-1 genome. The HIV-1 genome is composed of nine genes and is flanked on each side by the HIV LTR. Two of these genes, namely gag and env, code for structural proteins, pol codes for the enzymes of the virus, tat and rev for regulatory proteins, and vif, vpr, vpu and nef for accessory proteins.

1.1.3 HIV structure

The two molecules of RNA forming the HIV genome, which serve as genomic template for the next infected cell, are packaged into the conical viral capsid (composed of several copies of the p24 protein) together with the nucleocapsid protein p7, the reverse transcriptase, the protease and the integrase. The capsid is surrounded by a matrix (composed of the protein p17), which is surrounded itself by a host cell-derived envelope carrying cellular proteins and viral glycoproteins including the HIV envelope glycoprotein (gp)160 which consists of the transmembrane gp41 and the docking gp120[16-19] (Fig. 2).

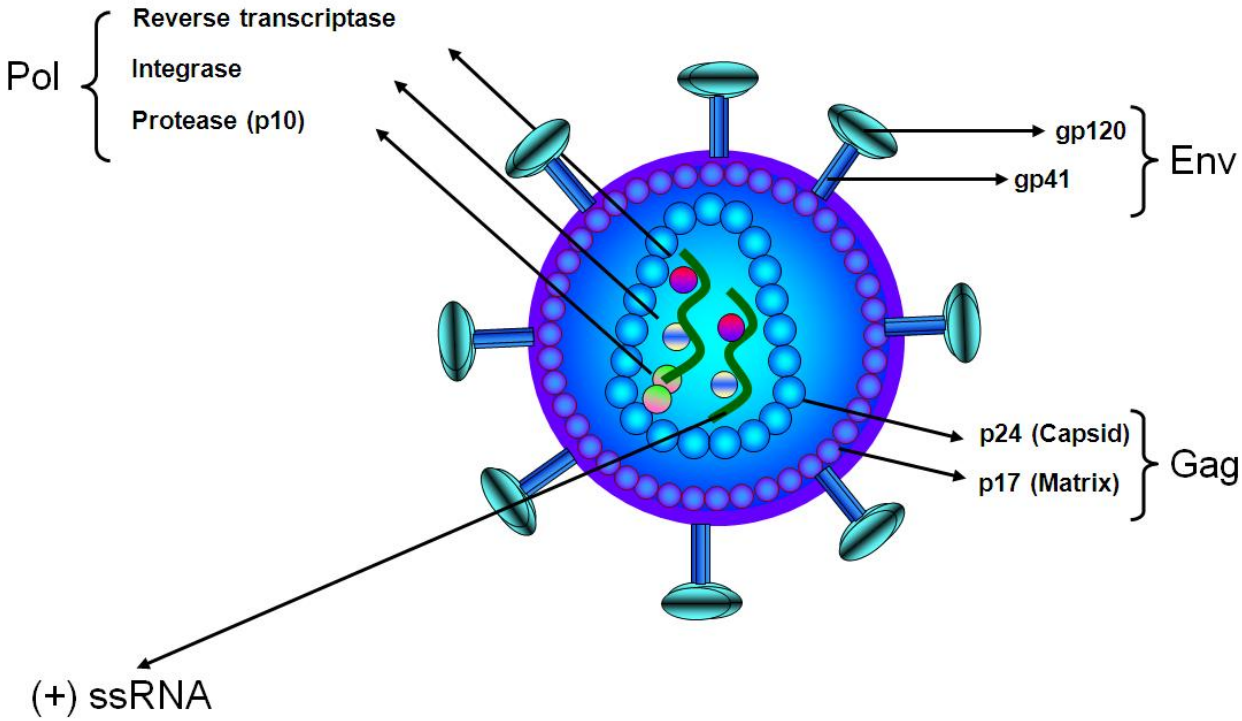

Fig. 2. The HIV-1 viral particle. HIV-1 virion consists of a capsid in which two RNA copies are packaged with the integrase, protease and reverse transcriptase, surrounded by a matrix and a lipid membrane originated from the host cell containing the HIV envelope gp120/gp41.

1.1.4 HIV replication cycle

HIV entry into cells requires the expression of the CD4 cell surface molecule[20] and a co-receptor, either CCR5 or CXCR4[21-25]. Binding of the HIV Env gp120 to CD4 results in a conformational change of gp120, which in turn facilitates the binding to the co-receptor[26-28]. This allows the HIV Env gp41 to fuse with the cellular membrane leading eventually to cellular entry[29].

CCR5-tropic (R5-tropic) strains are the strains primarily transmitted and also the strains which predominate until late stage disease (reviewed in[30]). Accordingly to the receptor complex HIV needs for cellular entry, HIV has a particular tropism for memory CD4+ T cells, macrophages, dendritic cells (DC) and monocytes, which express the CCR5 co-receptor[24].

In some cases, CXCR4-tropic (X4-tropic) strains, emerge in late stage disease [31, 32], with a tropism for naïve T lymphocytes[33], dendritic cells, Langerhans cells, monocytes, and CD34+ progenitor cells[33-35].

Subsequent to cell entry, HIV disassembles, reverse transcription ensues generating a double-stranded DNA from the HIV RNA[36-38]. To do so, the HIV own RT generates a complementary DNA (non-coding DNA) from the HIV RNA template. Subsequently, the RT which in addition to the RNA-dependent DNA polymerase activity has also ribonuclease activity (RNase H activity), degrades the HIV RNA [39, 40] and finally it synthesizes the other DNA strand (coding DNA) with its DNA-dependent DNA polymerase activity resulting in a double-stranded DNA[41].

The HIV DNA in concert with cellular proteins (i.e. importins) and HIV proteins (matrix, Vpr, integrase) will then form the pre-integration complex (PIC). The association of cellular and viral proteins with the viral DNA impedes the degradation of the latter and makes its integration into the cell genome possible. The HIV DNA integrates in a random position and its integration is much favored when the infected cell is activated. Once integrated, the HIV DNA is then called “provirus” (reviewed in[42]). The provirus will remain in the cell genome as long as the infected cell lives. This ability to remain latent in the cells is one of the main reasons why patients will not get cured although ART is very efficient to suppress HIV replication. For instance, the half-life of the latent reservoir is around 44 months, which means that for 10^5 latently infected cells, it would take 60 years until all these latently infected cells die[43, 44]. Thus, it is unrealistic that ART alone will able to eradicate HIV from an infected person and thus result in a cure. Moreover, interruption of ART results inevitably in HIV rebound from the viral reservoir.

When the cell is productively infected, HIV hijacks the cellular machinery to induce transcription of its gene products in order to produce progeny viruses. Cell activation goes along with up-regulation of NF-κB which is the primordial transcription factors binding to the LTR and driving HIV replication [45, 46]. Initially, fully spliced mRNAs are generated that code for the Tat, Rev and Nef proteins: Tat increases the rate of transcription and allows for the transcription elongation of a full-length genomic RNA[47, 48]; Rev regulates the export of unspliced HIV RNAs (Gag-Pol and Env RNA) from the nucleus to the cytoplasm, in combination with a specific cis-acting sequence for its function, called rev-response element,

which is a 520 bp fragment located in the env genomic region[49-51]; otherwise, without Rev, no single spliced or unspliced mRNA is exported which encode for the structural proteins Gag and Env and the HIV genome which will be incorporated into new virions. Finally, Nef plays a multitude of roles increasing HIV pathogenesis[52].

The structural HIV proteins will be post-transcriptionally modified either prior to assembly or within the immature virions; in particular, the Env polyprotein, into the envelope glycoproteins, gp41 and gp120[53, 54]. Similarly, the structural proteins which form the capsid, nucleocapsid and matrix, are encoded by a precursor protein that is processed (Gag p55) by the protease. Subsequently, the p55 and the Gag-Pol polyprotein (p160) are transported to the plasma membrane where HIV particles are assembled for viral budding[55, 56]

The HIV particles released have yet to mature for being infectious. Maturation is performed by the HIV protease, which cleave the polyproteins into functional structural proteins or active enzymes[56] (Fig. 3).

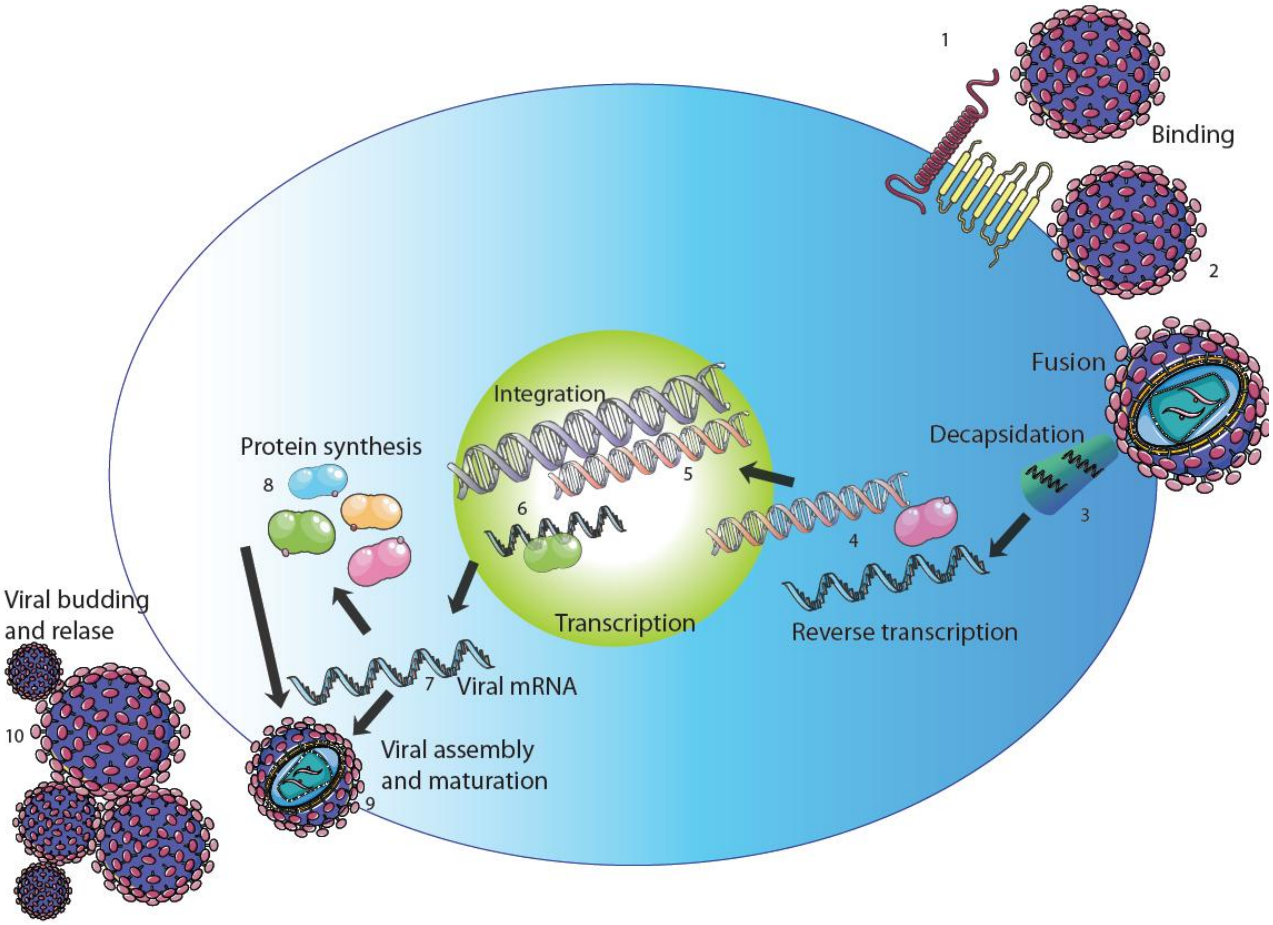

Fig. 3. The HIV replication cycle. HIV starts its replication cycle by binding (1) to the cell surface molecule CD4 and a co-receptor (CCR5 or CXCR4) (2) for cellular entry (fusion and decapsidation) (3). Key steps are reverse transcription (4) of the ss HIV RNA into DNA and its integration (5) into the host chromosome. Integrated proviral HIV DNA serves as template for the transcription (6) and translation of viral proteins (8) or to generate the HIV RNA (7) for its incorporation into newly produced viral particles (9). The structural viral proteins and the viral genomic RNA, together with the integrase, protease and RT, are assembled at the plasma membrane, where the budding (10) of the viral particles takes place and a new replication cycle starts (adapted from[57]).

1.1.5 HIV sequence evolution

A particular and very important property of HIV is its viral diversification within the host. In the current paradigm, transmission occurs with one or at most a few founder viruses within a

very short time frame viral diversification takes place generating thousands of distinct HIV strains (quasi-species). This viral diversification is due to i) the lack of proofreading activity of the RT[58, 59], ii) recombination events and iii) hypermutation by the APOBEC3 system.

- The lack of proofreading of the RT makes the process of reverse transcription highly failure-prone: it results in 1 error for each 10'000 to 100'000 nucleotides incorporated[60, 61]. Since 10^8 virions are produced every day[62], it means that there are 10^3 to 10^5 mutations produced every day in the viral population. Thus, several mutations on the HIV genome appear in a very short time frame in every single patient.

- The recombination occurs when a cell is infected at the same time by at least two different HIV strains or different viruses[63]. During reverse transcription in the next replication cycle, the RT "jumps back and forth" among the two RNA molecules, which produces a recombinant viral DNA[64]. Consecutively, all virions produced by this recombinant viral DNA will harbor the recombinant genotype[64].

- APOBEC3G/F acts as restriction factors by hypermutating the HIV genome (G-to-A mutations)[65, 66]. Although the accessory protein Vif generally counteracts APOBEC3G/F by sending them to the proteasome for their degradation[67], some Vif variants, partially neutralize these enzymes, promoting viral sequence diversification and increased pathogenesis[68, 69]. The role of APOBEC3G/F in the viral sequence evolution will be described more in details in the sections below.

The rapid viral diversification enables HIV to escape immune responses or drug pressure (immune escape variants, drug resistant HIV). It also represents a major hurdle for the generation of an efficient vaccine.

1.1.6 HIV infection

HIV is transmitted by body fluids (blood, semen, pre-ejaculatory fluid, vaginal secretions and breast milk[70]). The transmission modes are sexual intercourse, blood exchange, either by sharing needles or inadequate screening of blood donations, and mother-to-child transmission (vertical transmission)[71].

The main transmission worldwide is heterosexual intercourse (UNAIDS Global report 2012). Factors which increase the risk for transmission are i) mode of sexual intercourse with anal intercourse carrying the highest risk, ii) the height of plasma viral load in the HIV-positive partner and iii) presence of additional sexually transmitted infections such as Herpes simplex virus (HSV) mediated genital ulcers.

Vertical transmission takes place mainly during delivery, followed by breast milk-feeding and finally during pregnancy (*in utero*)[72]. Mothers which are efficiently antiretroviral therapy (ART) treated have a very low risk of transmitting the virus to their kids; indeed ART treatment is recommended before getting pregnant, or, in case of treatment-naïve pregnant women, it is recommended to start treatment at the beginning of the 2^{nd} trimester of pregnancy (EACS Guidelines, 2014).

During the delivery, if the viral load in the blood of the mother is elevated, it can potentially infect the newborn due to the blood contamination during partum. Additionally, caesarean operation decreases this risk by preventing the newborn to get in touch with the blood from the mother. Since HIV is present in the body fluids, including milk, breast-feeding is an additional transmission route for the newborn and therefore should be avoided[72]. However, some or most of these preventive procedures cannot be implemented in the third world countries for infrastructure or political acceptance or willingness[73-77].

HIV infection can be divided into three main phases: acute infection, chronic infection and AIDS. The first one refers to the primary infection, which defines the time period after transmission and up to three months after infection. While HIV infection is characterized by the presence of a large number of diverse strains, known as "quasi-species"[78], those HIV strains that are transmitted are only a few or even single viruses, also called founder viruses[79, 80]. Primary HIV infection presents as a flu-like disease which is not distinguishable from other viral diseases, with fever in >80% and rash in >50% of cases, oral ulcers, arthralgias, pharyngitis, malaise and diarrhea[81]. These symptoms manifest usually within the first 2-4 weeks after HIV transmission and may last a couple of days to some weeks (5-60 days). At this stage of HIV infection, the HIV RNA copy numbers in the plasma may peak with levels higher than 10^6 copies/ml[82-85], in parallel, the CD4+ T cell count drastically diminishes. As soon as HIV-specific CD8+ T cells are generated, the HIV RNA copy numbers decrease and the CD4+ T cell count recovers partially[86-88].

In the chronic phase following primary infection the patient is asymptomatic, however the disease continues: at around 3-6 months, the HIV RNA copy numbers (viral load) will reach a quasi-steady state called viral set-point[89]; this height of the viral set-point predicts the disease progression rate, which is monitored in clinical practice by measuring the CD4+ T cell number over time[90].

In average there are 800-1200 CD4+ T cells/µl of blood in a healthy person[91]. Some immunodeficiency is observed at CD4 T-cell numbers below 500 cells/µl, with opportunistic infections such as candidiasis, Kaposi sarcoma (caused by Human Herpes Virus-8) and tuberculosis (reviewed in[92]). Severe immunodeficiency is present when CD4+ cells are < 200 cells/µl, with wasting syndrome and opportunistic infections, such as extensive or disseminated Herpes, Cytomegalovirus (CMV) infection, cerebral toxoplasmosis, pneumonia with *Pneumocystis jirovecii* (formerly known as *Pneumocystis carinii*), among many other diseases (reviewed in[93]). A patient loses between 70-250 CD4+ T cells/µl per year[94] and thus it takes on average around 10 years to reach the critical threshold of 200 CD4+ T cells/µl[90]. This stage is best known as AIDS. Without ART, the life expectancy of patients with AIDS is most likely less than two years (Fig. 4).

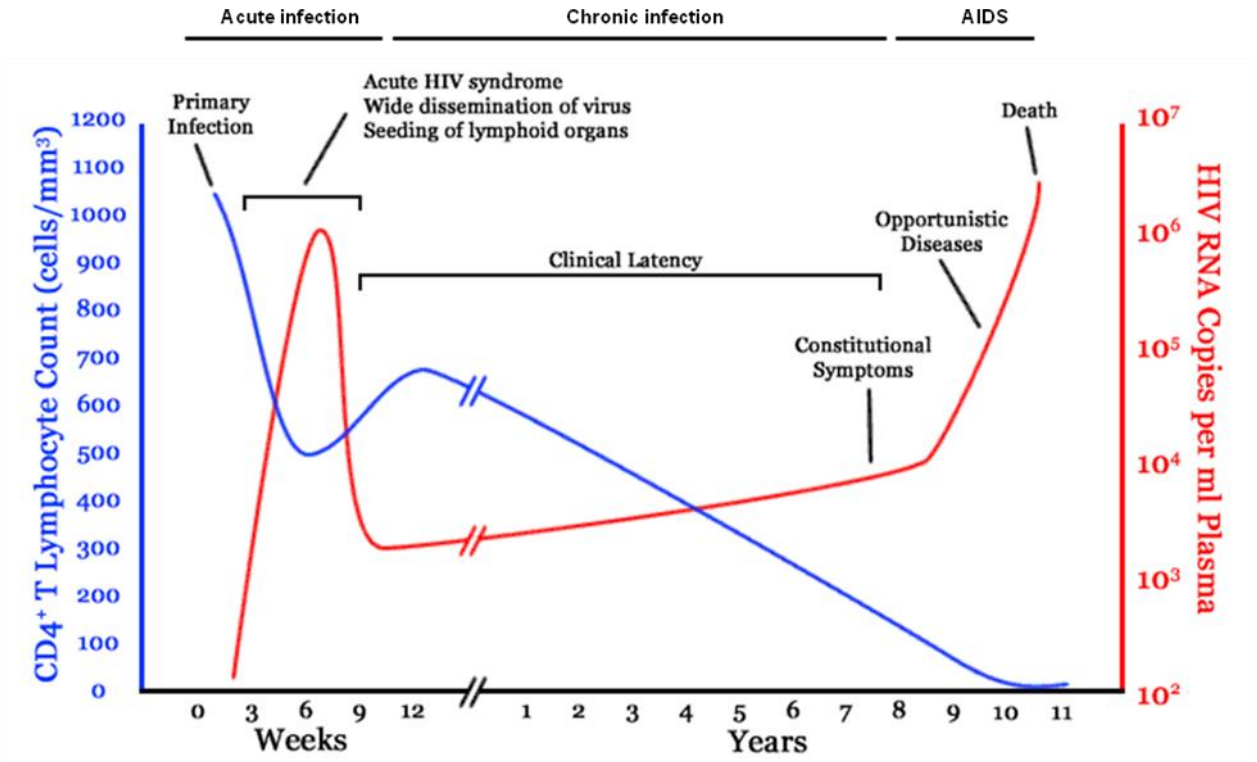

Fig 4. Natural HIV infection depicting the acute infection, the chronic infection and the AIDS stage (adapted from[95]).

In 1996 first data were reported from HIV+ patients treated by a regimen consisting of two nucleoside analogues combined with a protease inhibitor: this antiretroviral treatment (ART) showed a dramatic decrease of the HIV viral load and a substantial increase in the CD4+ T cell counts[96, 97]. Moreover, a new drug class, non-nucleoside analogues, was also introduced in 1996[98, 99]; most recently, integrase inhibitors were approved for the clinics in 2007[100]. Today, the clinician has a choice of 34 drugs which may be differentially combined[101], e.g., two nucleoside analogues (13 different ones) and either a non-nucleoside analogue (6 different ones), a protease inhibitor (11 different ones) or an integrase inhibitor (2 different ones), reducing viral replication almost completely and increasing the recovery of the immune system (Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents, 2014).

Thus, HIV infection is not anymore the deadly disease as in the eighties and early nineties, at least in the wealthy countries. However, the widespread use of ART also revealed disadvantages such as adverse events, emergence of resistant strains in the case of poor adherence, psychological dependence and life-long intake. Very importantly, ART was not successful to stop the HIV pandemic.

1.2 IFN-α-induced cellular restriction factors and HIV-1 accessory proteins

Authorship contributions

I wrote all the review and did all the figures and tables present. Annette Audigé and Roberto Speck corrected the text and the figures.

Introduction

The human immunodeficiency virus (HIV) type-1 belongs to the lentivirinae, a sub-family of the retroviridae family, which are single-stranded RNA viruses. Common to all members of the retroviridae family are the structural genes capsid (gag), polymerase (pol), and envelope (env), encoding the Capsid and the Matrix; the enzymes, i.e., Reverse Transcriptase, Protease and Integrase; and the Envelope, respectively[1-3]. The lentiviridae are also named complex retroviridae because they have regulatory or virulence genes in addition to the structural genes. HIV-1 is the prototype lentivirus with six additional genes, two coding for the regulatory proteins Rev[4] and Tat[5] and four for the so-called "accessory proteins" Vif (acronym for viral infectivity)[6], Vpr (for viral protein R)[7], Vpu (for viral protein U)[8] and Nef (for negative factor)[9].

The term "accessory proteins" is misleading: it dates back to cell culture experimentations where the accessory proteins, according to the cell model used, were dispensable for HIV-1 replication[10-13]. In fact, HIV-1 strains deficient in a distinct accessory protein show a dramatic decrease in replication capacity or even only abortive infection *in vivo*. Each accessory protein has its individual role(s) in HIV-1 replication: Vif increases HIV's infectivity[6], Vpr amplifies its rate of replication and cytopathic effects[7], Vpu enhances its viral production and transmission[14], and finally Nef increases HIV-1's infectivity[12, 15]. All these effects of the accessory proteins occur through interfering with or exploiting host cellular proteins (reviewed in[16]).

On the one hand, HIV-1 hijacks cellular proteins required for viral replication in each single step, from viral entry[17-19] to viral production[20]. The host, on the other hand, interferes with HIV-1 replication at different steps in the HIV-1 replication cycle with its antiviral proteins (reviewed in[21]). These antiviral proteins are also called HIV-1 restriction factors.

They are constitutively expressed and/or upregulated by interferon (IFN). IFNs belong to the first cytokines secreted subsequent to invasion by pathogens and have manifold, very potent anti-viral effects[16]. To replicate efficiently despite the IFN response, HIV-1 has developed different counter-strategies.

This review focuses on the IFN-α-mediated anti-HIV mechanisms and the counteracting strategies employed by HIV-1, in particular of APOBEC3G/F and Vif , Tetherin and Vpu, SAMHD1 and Vpx, and finally TRIM5α and the Capsid protein. Table 1 shows the main IFN-stimulated restriction factors against HIV-1, indicating their antiviral function and their upregulation by the different IFN types.

Table 1. Main anti-HIV restriction factors and their upregulation by IFN.

Host restriction factor	Restriction mechanism	IFN upregulation
APOBEC3F/G	Hypermutation of the nascent cDNA, other mechanisms	IFN-α[22-24]
Bst-2/Tetherin	Tethering of viral particles to the cell surface for subsequent endocytosis and degradation	IFN-α[25, 26]
CNP	Binding to Gag, blocking viral particle assembly	IFN Type I[27, 28]
HERC5	Arresting Gag particles at the assembly site on the plasma membrane.	IFN-β[29]
IFITM 1, 2, 3	Inhibition of viral entry, negative effect in Gag expression	IFN type I and II[28, 30]
ISG15	Inhibition of viral budding and release	IFN-α[31]
MX2	Inhibition of nuclear import	IFN-α[32, 33]
PKR	Inhibition of protein translation	IFN-α[34]
Rnase L/2-5A	Viral RNA degradation	IFN type I and II[35, 36]
SAMHD1	Depletion of dNTP pool during reverse transcription	IFN-α and IFN-γ[37, 38]
SLFN11	Inhibition of viral protein synthesis, codon-usage-dependent manner	IFN Type I[39, 40]
TRIM5α	Premature uncoating of	IFN type I and II[41,

	the viral capsid or capsid degradation	42]
TRIM22	Transcription inhibition, alteration of Gag trafficking	IFN type I and II[43]

IFN-α in HIV-1 infection

IFN-α signaling

IFN type I comprises IFN-α as well as IFN-β, -ω, -ε, -κ, -δ, -ξ and –τ (reviewed in[44, 45]). There are 13 isoforms of IFN-α in humans with 75 to 99% of amino acid homology, which are encoded by at least 14 different genes in chromosome 9. IFNs are produced and released upon the stimulation of cells by structural motifs of pathogens containing so-called pattern recognition motifs. These pattern recognition motifs are present in double-stranded RNA (dsRNA), double-stranded DNA (dsDNA), DNA:RNA hybrids and single stranded RNA (ssRNA) from pathogens and are recognized by toll-like receptors (TLR) 3, 7/8 and -9, cytosolic sensors like RIG-I, MDA5[46-48], LPG2 [49], DAI[50] and IFI16[51, 52]. Additionally, IFN type I production is enhanced by cytokines like interleukin-4 (IL-4), -7 and -15[53].

Plasmacytoid dendritic cells (pDCs) are the main producers of IFN-α upon viral infection, although almost all cells are capable of producing IFN-α[54-57]. Once IFN-α is produced and binds the type I IFN receptor complex on a target cell, it triggers the dimerization of the interferon alpha receptor (IFNAR) 1 and 2, leading to the activation of the JAK/STAT pathway[58]. It eventually culminates in the translocation of the IFN-stimulated gene factor 3 (ISGF3) complex to the nucleus and the transcription of hundreds of IFN-stimulated genes (ISGs). There are other "non-canonical pathways" of IFN-α signaling that also induce ISGs (reviewed in[59]). These include (i) the v-crk sarcoma virus CT10 oncogene homolog (avian)-like (CRKL) pathway, which binds to the IFN-γ–activated sites (GAS)[60, 61], (ii) the phosphoinositide 3-kinase (PI3K) pathway and (iv) the NF-κB cascade which result in the upregulation of pro-survival signals and increase of many GTP-binding proteins[62, 63], and finally (iv) the mitogen activated protein kinase (MAPK) pathway, which is important for antiviral properties of IFN-α, regulation of cell growth and cellular differentiation[64, 65].

Most of the ISGs identified so far have multiple functions including host defense and in particular antiviral defense, immune modulation, inflammation and apoptosis but also

functions unrelated to the immune system, such as metabolic enzymes activation, adhesion, transcriptional factor and cell signaling[66].

IFN-α sets the cell in an antiviral state, i.e., a subset of the ISGs is active against the invading pathogen or interferes with the replication cycle by blocking key cellular functions. Among the multiple IFN-α-mediated antiviral effects, the best studied ones include inhibition of viral and cellular translation[67-69], induction of apoptosis[70, 71], degradation of both cellular and viral RNA[72, 73] and inhibition of nuclear import[74].

IFN-α signaling also results in activation of DCs[75], natural killer (NK) cells [76], B cells[77, 78] and naïve T cells[79]. Additionally, IFN-α leads to MHC class I and II upregulation, which subsequently activates CD8+ and CD4+ T-cells, respectively, making a link between the innate and the adaptive immune system[80].

IFN-α signature in HIV-1 infection

As stated above, pDCs are the main producer of IFN-α. HIV-1 induces IFN-α production by infecting pDC precursors[81], or by infecting directly pDCs[82-84]. A productive infection is not required for IFN-α induction since the recognition of HIV-1 ssRNA by either TLR7 or 9 subsequent to HIV-1 particle endocytosis triggers the production of IFN-α. Binding or fusion does not result in any IFN-α production[84]. It was recently reported that ssDNA from HIV-1 stimulates IFN-α expression in primary macrophages via its sensing by the IFN-inducible protein 16 (IFI16)[85]. Knockdown of IFI16 decreases the expression of several ISGs and increases viral infection in macrophages. However, these experiments were conducted by transfecting ssDNA into macrophages. Data so far from productively HIV-1-infected monocyte-derived macrophages (MDMS) do not support that this mechanism is operative *in vivo*, since HIV-1 infection of macrophages does not lead to IFN-α production[86].

Similarly to macrophages, HIV-1 infection of CD4+ T-cells does not result in any IFN-α production[87]. Several potential mechanisms may explain this phenomenon: i) lack of TLR7 and 9 expression in CD4+ T-cells, ii) IFI16, which senses incomplete DNA reverse transcripts (dsDNA), abundant in abortively infected cells, like resting CD4+ T-cells[52]. This sensing leads to the activation of caspase-1, which in turn induces a fulminant cell death of these cells through a process called pyroptosis iii) SAM domain and HD domain-containing protein 1 (SAMHD1) (described in details below) prevents IFN-α production in CD4+ T-cells and

myeloid cells by blocking infection during reverse transcription, preventing HIV-1 DNA accumulation[88, 89]. However, the Vpx protein of HIV-2 and of several simian immunodeficiency viruses (SIVs) is able to degrade SAMHD1 and rescue infection in the myeloid cells[90], inducing IFN-α production[91] and iv) Three prime repair exonuclease 1 (TREX1), which digests excessive proviral HIV-1 DNA after reverse transcription of the viral RNA[87]. TREX1 is present in macrophages[92], as well as B-cells and T-cells[93]. In the latter two cases, the proviral DNA could theoretically be sensed and stimulate IFN-α production.

The IFN-α produced by the pDCs results in a clear IFN-α signature with the upregulation and downregulation of hundreds of genes *in vitro* and *in vivo* in CD4+ T-cells and macrophages (70% of upregulated and 30% downregulated) and reduces viral replication in CD4+ T-cells[94]. This IFN-α signature has been associated with hyper-immune activation of the CD4+ T-cells, leading to apoptosis[95], as well as the induction of apoptosis as a direct effect of IFN-signaling[70, 71]; both mechanisms may contribute to the progressive CD4+ T-cell depletion. Table 2 summarizes ISGs reported concomitantly in 4 different publications (Table 2).

Table 2. Most common ISGs.

ISG	GenBank accession number	Audigé et al[94].	Rotger et al[96].	Sedaghat et al[95].	Woelk et al[97].
ADAR	AB209891		x		
APOBEC3F	JF262037	x	x		
APOBEC3G	BC024268	x	x		
APOBEC3H	BC069023		x		
BST2	BC033873	x	x		
CCL2	M26683				x
CCL8	Y16645				x
CIG5	AF026941				x
DDX58	BC136610	x	x		
EIF2AK2	BC101475		x		
G1P2 (ISG15)	AA203213	x		x	x
GBP1	BC002666	x			
IFI6	BC015603	x	x	x	x
IFI16	BC017059	x			
IFI27	BC015492			x	
IFI35	BC001356	x	x		
IFI44	BC022870	x		x	
IFI44L	BC015932	x		x	

IFIH1	BC111750	x	x		
IFIT1	M24594	x	x	x	x
IFIT2	BC032839	x			
IFIT3	BC001383	x	x	x	
IFIT4	AF026939				x
IFIT5	BC025786	x			
IFITM1	J04164	x	x	x	x
IFITM3	X57352	x			x
IFNAR1	BC021825		x		
IFNAR2	BC002793		x		
IFNG	BC070256		x		
IFNGR1	BC005333		x		
IFNGR2	BC003624		x		
IFRG28	BC013161	x			
IRF1	BC009483		x		
IRF7	BC136555	x		x	
IRF9	BC035716	x	x		
ISG15	M13755		x		
ISG20	BC000575	x			
JAK1	AB219242		x		
JAK2	BC039695		x		
MX1	M33882	x	x	x	x
MX2	M30818	x			x
OAS1	X04371	x	x	x	x
OAS2	BC069052	x	x	x	
OAS3	AF063613	x	x	x	
OASL	BC117410		x	x	
PIAS1	BC118587		x		
PLSCR1	AB006746	x			x
PSMB8	BC001114		x		
PTPN2	BC016727		x		
RNASEL	BC115697		x		
RSAD2	BC017969	x			
SOCS1	AF132440		x		
SP100	AF056322	x			
STAT1	BC002704	x	x	x	
STAT2	BC051284	x	x		
TAP1	L21207		x		
TRIM22	BC035582	x	x		
TRIM5	BC021258		x		
TYK2	BC014243		x		
ZBP1	BC131706		x		

The functions of most of these ISGs are unknown (Table 2). For some of them, notably APOBEC3G, Tetherin, and TRIM5α, their mechanism of action against HIV-1, other retroviruses and enveloped viruses has been discovered recently (see below).

IFN-α: good or bad in HIV-1 infection

IFN-α plays an important antiviral role during acute HIV-1 infection. This is remarkable since there is an increase of IFN-α in the peripheral blood, immediately after the initial viremia is detectable[98]. Additionally, there is an upregulation of hundreds of ISGs, with antiviral effects (see below). Interestingly, IFN-α decreases viral replication of founder viruses during initial steps of transmission and only the most IFN-α-resistant viruses succeed to establish a productive infection[99]. Finally, in acute SIV infection there is a negative correlation between peak IFN-α levels and viral loads[100] or in African Green Monkeys, a strong and rapid IFN-α response contributes to the control of non-pathogenic SIV_{AGM}[101].

Moreover, CD8+ T cells, in elite controllers or asymptomatic infected people, produce IFN-α, which is thought to contribute to the protracted or absent progression rate and which might act by up-regulating ISGs in CD4+ T-cells and in an autocrine fashion by increasing the CD8+ T cell non-cytotoxic antiviral response[102]. Additionally, elite controllers have increased levels of a particular ISG, namely, the restriction factor Schlafen 11[103].

Although the number of circulating pDCs decreases during HIV-1 infection, the pDCs that survive are hyperfunctional, producing large amounts of IFN-α[104], resulting in increased IFN-α concentrations as quantified in the peripheral blood[105, 106]. This increase is thought to be deleterious. For instance, *in vitro*, HIV-1 infection is responsible for IFN-α production, which in turn induces TLR7 activation. This TLR7 stimulation transforms pDCs into DCs expressing the TNF-related apoptosis-inducing ligand (TRAIL)[107, 108]. *In vivo*, TRAIL-expressing DCs induce apoptosis of CD4+ T-cells in patients, leading to CD4+ T-cell loss[109]. Nevertheless, in elite controller patients, this TRAIL induction is useful to kill infected CD4+ T-cells[110], which in that case represents a protective effect of IFN-α. Moreover, sustained TLR7 stimulation by HIV-1 ssRNA itself may contribute to HIV-associated immune activation[111].

Although IFN-α plays an important antiviral role for the initial phase of immune defense, as mentioned above, it is controversial whether exogenous administration of IFN-α as an alternative or complementary treatment for HIV-1 infection is good or bad. *In vitro* treatment with IFN-α inhibits HIV-1 replication in T-cell lines like CEM-174[112] and MT4R5[113], as well as in primary CD4+ T-cells[94, 114, 115]. In macrophages (*ex vivo*), a high restriction of

HIV-1 by IFN-α was documented at early stages of viral infection[116]. Also, expression levels of several cellular factors, including TRIM5α, CypA, APOBEC3G, SAMHD-1, TRIM22, Tetherin and TREX-1, and different anti-HIV miRNAs, which were increased by IFN-α, were considered to be partially responsible for this inhibition[117].

In vivo, IFN-α treatment has been studied mainly in HIV-1/Hepatitis C virus co-infected patients with very prominent antiviral effects[118-120]. In mono-infected ART-naïve patients or patients which were off ART for at least 12 weeks, IFN-α decreased the viral load by a factor of approximately 10[121-123] and upregulated several host restriction factors with known activity against HIV-1[124] (see below). IFN-α treatment during acute HIV-1 infection also enhances the production of anti-HIV-1 antibodies, improving control of viral replication[125], highlighting the importance of this cytokine during primary HIV-1 infection. In patients, it significantly enhanced CD8+ T cell activation, although no CD4+ T cell activation was detected[126].

The protective anti-HIV-1 effects of IFN-α have also been corroborated in humanized mice, with a complete suppression of viral load due to IFN-α treatment[127]. On the other hand, IFN-α appears also to have disadvantageous effects: for instance, IFN-α upregulates the CCR5 co-receptor in the T-cell precursors in the thymus of humanized mice[128] or human CD4+ T-cells *in vitro*[129], which increases the susceptibility of the cells to HIV-1 infection, leading to increased cell depletion. Additionally, in humanized mice, IFN-α treatment increased CD4+ and CD8+ T-cell activation[130].

In vitro, adding neutralizing antibodies against IFN-α enhanced viral replication but inhibited CD4+ T cell depletion[129], and TRAIL upregulation and immune activation were decreased[131], which indicates that IFN-α increases immune activation and CD4+ T-cell depletion. IFN type I treatment as studied *ex vivo* in PBMCs results in the death preferentially of effector memory CD4+ T-cells by increasing the expression of a pro-apoptotic protein called Bak[100]. Increased expression of Bak acts by activation of the CD95/Fas-mediated apoptosis pathway. In infected patients, there was a positive correlation between ISGs expression, Bak levels, apoptosis, viremia and a negative correlation with CD4+ T-cell counts[100].

One approach to counteract the IFN-induced immune activation is to block or decrease IFN-α production. In two previous clinical trials, 27 HIV-1 infected patients (16 ART naïve and 27 ART-treated) and 122 HIV-1 patients (ART-naïve and ART-treated) were vaccinated against IFN-α, using an albumin-free preparation of recombinant human IFN-α-2b, chemically inactivated, as an immunogen, producing anti-IFN-α antibodies[132, 133]. These studies showed promising results, including stabilization of CD4+ T-cell counts, stable viremia over time and decrease of IFN-α titers, as compared to non-vaccinated patients.

Finally, IFN-α plays a major antiviral role inhibiting the cross-species transmission of primate lentiviruses, which is more pronounced than the antiviral effect against lentiviruses adapted to their natural host[134]. Very interestingly, this effect was not due to the known restriction factors TRIM5α, APOBEC3, Tetherin, or SAMHD1 (described in details below), indicating the presence of undiscovered restriction factors which play a major role in blocking the cross-species transmission of HIV-1 and other lentiviruses.

Taken together, it appears that IFN-α has two faces like Janus, the Roman god ; one face of IFN-α is its inhibition of HIV-1, especially during acute infection, and the other face is its detrimental effect on CD4+ T-cells, increasing their immune activation and depletion, during chronic infection.

APOBEC3G/F and Vif

Discovery of APOBEC3G/F

The Vif protein of HIV-1 is a 23 kDa protein constituted by 192 residues[135]. It was from the beginning of its discovery postulated to have an important role on HIV-1 infectivity and to be essential for viral spread[6]. The reason for this was unclear, mainly because this protein was non-essential in some cell cultures but not in PBMCs[136, 137] or *in vivo*[138], but dispensable in some cell lines. This enigma was solved when a novel restriction factor initially called CEM15 was identified[139]. In that work, the researchers limited the numbers of potential factors by subtraction hybridization of PCR-amplified fragments from cells permissive or non-permissive for replication of Vif-deficient HIV-1. The resulting PCR products were used for generating a cDNA library, which then served, as probes against RNAs extracted from permissive and non-permissive cell lines. By this approach, a transcript of 1.5 kb was identified only present in the non-permissive cell lines. This transcript, CEM15,

belongs to the family of deaminase enzymes of the apo-lipoprotein B editing complex 3 (APOBEC3), and was subsequently renamed APOBEC3G (A3G)[140, 141]. Soon after its discovery, APOBEC3F (A3F), another protein of the same family, was found to have similar effects on HIV-1 and HIV-2[142, 143]. These proteins are constitutively expressed in several cell lines (CEM, MT2, PM1, etc.)[144, 145] and in primary cells like CD4+ T-cells, macrophages and DCs[146].

APOBEC3G/F and IFN-α

A3G/F are prototype ISGs. *In vitro*, they are upregulated by exogenous IFN-α, although the level of upregulation depends substantially on the cell type and the donor. For instance, A3G is upregulated in primary hepatocytes, MDMS and pDCs (where it is thought to exert anti-HIV activity)[24] by IFN-α manifold in *ex vivo* samples from healthy donors[22], but not at all or very weakly in primary CD4+ T-cells, PBMCs or in the CD4+ T-cell line H9. Of note, all the latter cells express high basal levels of A3G[23]; in fact, downregulation of A3G in MDMS or MDDCs (monocyte-derived dendritic cells) with siRNAs goes along with loss of the antiviral effect[22, 24]. Likewise, A3G is also upregulated by IFN-β in MDMS[117], neuronal cells/astrocytes[147] and, very weakly, in PBMCs[148]. Similarly to A3G, A3F is upregulated by IFN-α in MDMS, MDDCs and hepatocytes but not induced in primary CD4+ T-cells[149, 150]. No data concerning A3F upregulation upon IFN-β treatment is available. Moreover, the IFN-α-mediated upregulation of A3G/A3F is associated with a blocking of viral replication and a decrease of the transmission from MDDCs to CD4+ T-cells[150]. Likewise, the upregulation of A3G is associated with the inhibition of HIV-1 replication[117]. However, there are also several other factors upregulated at the same time, such as TRIM5α, Tetherin, TRIM22, SAMHD1, TREX-1 and hundreds of other ISGs whose induction by IFN-α was not determined in this study. Finally, all APOBEC3 deaminase family members are upregulated by IFN-α[150-152]

APOBEC3 family members

The APOBEC3 family includes seven members, APOBEC3A (A3A), APOBEC3B (A3B), APOBEC3C (A3C), APOBEC3DE (A3DE), APOBEC3F (A3F), APOBEC3G (A3G) and APOBEC3H (A3H). These proteins are encoded by seven genes, all arranged in tandem on the chromosome 22, and all have the capacity to mutate cytosines to uracils[153, 154]. From these seven members all have anti-HIV activity, but the really relevant ones are A3F and A3G

due to their potent negative effect on HIV-1 infectivity and levels of expression, which is much higher than that of the other members. Table 3 summarizes the antiviral activity, expression levels and Vif sensitivity of each one of the seven APOBEC3 members.

Table 3. APOBEC3 family members.

APOBEC3 family member	Antiviral activity	Expression level	Vif sensitivity
APOBEC3A	Human parvovirus adeno-associated virus (AAV), HIV-1 in myeloid cells and retrotransposons	High in monocytes, macrophages and neutrophils	Resistant[155]
APOBEC3B	Low anti-HIV activity	Low	Resistant[152]
APOBEC3C	SIV, very low anti-HIV activity	Middle	Sensitive[152, 156]
APOBEC3DE	Low anti-HIV activity	Middle	Sensitive[152]
APOBEC3F	Moderate anti-HIV activity	Middle	Sensitive[152]
APOBEC3G	Strong anti-HIV activity	High	Sensitive[139, 152, 157-159]
APOBEC3H	Low anti-HIV activity	Low	Resistant[152]

APOBEC3G/F antiviral mechanism

Production of viral particles goes along with the incorporation of A3G/F by its binding to the Gag protein and the HIV-1 RNA[160]. In the newly infected cell, A3G/F by virtue of their deaminase activity will mutate cytosines to uracils by removing the amino group present in the cytosine of the nascent negative HIV-1 DNA strand during reverse transcription[153, 154]. Uracil, as a thymine analogue, will result in the incorporation of A into the plus strand leading to G-to-A mutation in the proviral DNA. For A3G, this occurs in a GG-to-GA context and for A3F in a GA-to-AA context[157-159, 161, 162]. The A3G/F editing effect generates several non-synonymous mutations in HIV-1 DNA, leading to diversity of the virus but also

to many stop codons[163, 164] and thus in several truncated proteins[165, 166], which in turn inhibits viral spread and drastically decreases viral infectivity. For instance, infectivity of Vif-deficient viruses is reduced up to 97% and viral production up to 300 times in CEM cells, expressing basal levels of A3G[139].

While an entirely unopposed deployment of the A3G/F editing system is detrimental to HIV-1, a partially active A3G/F editing system may promote viral diversity, which is beneficial for viral evolution, immune escape and emergence of drug resistance[167-169]. Additionally, the truncated proteins, as a result of hypermutation or partial hypermutation, are an additional source of peptides which are potentially loaded onto MHC class I and II molecules, which links the innate and the adaptive immune system[165, 166].

The effects of A3G/F-mediated hypermutation is mainly observed on integrated HIV-1 DNA and to a lesser extent on intracellular viral RNA, but only rarely on plasma viral RNA[170, 171]. This might be explained by the fact that hypermutated viral genomes might code for many truncated proteins, resulting in no viral progeny (see above). Therefore, only infected cells with a non-hypermutated or partially hypermutated genome would be able to produce virions. The "hypermutation" phenomenon of A3G/F was also found for other retroviruses[159] and the hepatitis B virus (HBV)[172, 173]. Of note, A3G/F are highly expressed in hepatocytes (see above), where the HBV replicates and thus can be inhibited by these factors.

A3G/F may interfere with viral replication independently of hyperamination by inhibiting the reverse transcription elongation; this was shown in cell-free assays where reverse transcripts were measured by qPCR and elongation was blocked[174] or using an artificial mutant of A3G on its catalytic domain (C291S) and again a blockage of transcription elongation was observed[175]. The mechanism remains unknown; thus, this non-editing effect of A3G/F impedes the synthesis and accumulation of viral cDNA[174, 175]. Of note, such catalytic domain-mutant is an artificial construction by site-directed mutagenesis and has not been reported *in vivo*. The same mechanism is also operative for HBV and human T cell leukemia virus type 1 (HTLV-1)[145, 176, 177].

It has been reported previously that the accessory protein Vpr activates the DNA damage stress-sensing response and promotes G2 cell-cycle arrest, which results in the upregulation of

several NK cell-activating ligands (ULBP1, ULBP2, ULBP3, etc.), which increase the lysis of infected cells by NK cells[178-180]. On the other hand, A3G might also contribute to this process. The hypermutation or partial hypermutation of HIV-1 by A3G results in a significant incorporation of uridine[181]. The HIV-1 Vpr accessory protein, together with a cellular uracil glycosylase, decreases the uridine accumulation[181]. However, this mechanism activates the DNA-damage-response pathway, which in turn upregulates NK cell-activating ligands (in this case, ULBP1)[181]. Although Vpr is produced in the infected cells, and the viral cDNA is deaminated just at the beginning of the viral replication cycle, partially hypermutated cDNA might still be able to produce viral progeny[168], including Vpr and several other viral proteins. However, the contribution of A3G in this process remains to be confirmed in more physiological studies such as *in vivo* studies.

APOBEC3G/F neutralization by Vif

HIV-1 Vif counteracts the A3G/F editing system by promoting their polyubiquitination and proteasomal degradation involving the cullin 5-containing E3 ubiquitin ligase, composed of cullin 5, elongin B, elongin C and a RING-box protein[182-184] (Fig. 1). Vif can directly bind A3G/F using its N-terminal region, thereby serving as an adaptor between A3G/F and the ubiquitin ligase complex (Fig. 1) [162, 182-185]. The transcription factor core binding factor β (CBF-β) is essential for the Vif-E3 ubiquitin ligase complex - without CBF-b there is no proteasomal degradation[186-188]. Vif binds the amino acids 126-132 in the N-terminal region of A3G, while it binds the residues 283-300 of the C-terminal region of A3F[189]. Of note, the anti-A3G/F effect of Vif is species-specific[190-193].

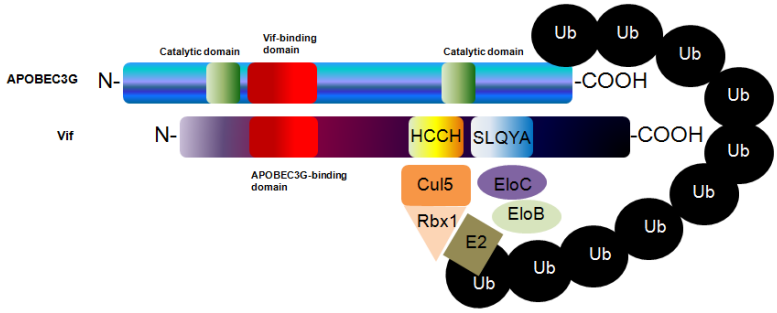

Figure 1. Vif binds APOBEC3G for its proteasomal degradation. APOBEC3G possesses 2 catalytic domains, necessary for its deaminase-dependent antiviral activity. Close to the first catalytic domain, there is a region where Vif recognizes and binds it (Vif-binding domain).

Vif, on the other hand, has an APOBEC3G-binding domain, used to bind APOBEC3G and target it to the proteasome. This is done by recruiting the Cul5 protein, which in turn is bound to Rbx1 and E2 and the SLQYA recruits the EloC and EloB. This results in a polyubiquitination of APOBEC3G and its proteasomal degradation.

In vivo studies of APOBEC3G/F

It is difficult to assign a specific anti-HIV-1 effect to the A3G/F system in HIV-1 infected patients since all studies rely on correlations; there are no compounds available which would permit to interfere with the A3G/F–Vif axis so far.

For instance, in a clinical study with 30 HIV-infected patients, A3G and A3F mRNA levels increased after HIV-1 infection (between primary infection and chronic infection)[140]. When comparing the mRNA levels of A3G/F from PBMCs in HIV-infected patients with a low or a high viral set point as defined by 6-30 months after primary infection (in this study), the mRNA levels of A3G and A3F were higher in people with a lower viral set point 22 months post infection. Of note, the viral set point determines the HIV-1 progression rate[194]. To what extent the levels of A3G/F are a major determinant of the viral set point remains unknown. In ART-naïve HIV-Hepatitis C Virus (HCV) co-infected patients, anti-HCV treatment with IFN-α and Ribavirin led to an increase of A3G/F levels in PBMCs and an increase in viral hypermutation, mainly GG-to-AG mutations (A3G) but also GA-to-AA mutations (A3F) were observed, indicating an A3G/F-related editing effect *in vivo*[124]. In another clinical study involving long-term non progressors, a negative correlation between A3G gene expression level, extent of HIV hypermutation and control of viremia was observed, suggesting a protective effect of the A3G/F editing system[195]. Similarly, a negative correlation between the viral load and expression of A3G/F was observed in SIV-infected rhesus macaques with long-term non-progression [196].

However, other studies reported no obvious protective effects of the A3G/F editing system. For instance, in a clinical study in HIV-infected adults (ART naïve and chronically infected), the levels of potential A3G-mediated mutations found in integrated HIV DNA (vif, env and gag regions) did not correlate with the viral load[154] or in a similar study with ART-naïve or treated, chronically infected HIV-infected children the gene expression level (mRNA) of A3F

or A3G did not correlate with the editing effect (hypermutation events) and disease progression[197]. Interestingly, in this case the majority of hypermutations observed were in a GA-to-AA context (A3F). Thus, the clinical relevance of the A3G/F editing system remains to be shown. The highly polymorphic nature of A3G and A3H speaks in favor of the notion that there is a selective pressure on these genes and this in turn speaks in favor of an important role as a defense system against retroviruses or for a yet unknown function[198-200]. For instance, there is the H186R polymorphism in the A3G protein, whereby the 186R allele is associated with an increased CD4+ T cell-depletion and thus accelerated AIDS development in African Americans[201]. Puzzlingly, both polymorphisms displayed a preserved antiviral activity in cell cultures[201]. Also, different polymorphisms and splice variants of the A3H have been identified, which have variable antiretroviral activities and are mostly resistant to the neutralization by the Vif protein *in vitro,* for instance haplotype I (HapI), 18R/105G/121K/178E; or haplotype II (HapII), 18R/105R/121D/E/178D[202, 203]. In particular, the polymorphism K121E is responsible for binding and susceptibility to Vif[204].

Data from humanized mice demonstrate the significance of the anti-viral mechanism of A3G *in vivo*[205]. HIV-1 strains deficient in Vif did not replicate, since there was no detectable viral load in the plasma of the infected animals. Subsequent sequencing of proviral DNA from splenocytes of infected animals with HIV-1 wildtype (WT) revealed the fingerprints of deamination by APOBEC3 members. There was a predominant accumulation of G-to-A mutations, which were induced by different APOBEC3 members and not only A3G/F. Moreover, the hypermutation created mainly stop codons and not non-synonymous mutations that could potentially increase viral diversity.

Other functions of APOBEC3G/F

Other functions than being a very important intrinsic immune defense system has not yet been attributed to the APOBEC3 system. We would like to emphasize that this APOBEC system also protects the host cell against endogenous retroviruses and retrotransposons by introducing non-synonymous mutations in their genome and by decreasing their cDNA levels[206-209].

Tetherin and Vpu

Discovery of Tetherin

Three observations were essential for the discovery of the restriction factor Tetherin/Bst-2. First, infection with HIV-1 strains deficient in the Vpu gene (ΔVpu) resulted in reduced viral particle release in epithelial cell lines such as HeLa cells as compared with the monkey cells COS-7[210]. Heterokaryons between those two cell lines resulted in a restrictive phenotype, which pointed to a dominant factor provided by the HeLa cells[210]. Second, IFN-α treatment decreased the release of viral particles in 293T cells infected with HIV-1-ΔVpu more than expected compared to HIV-1 WT, which suggested that an ISG is specifically neutralized by Vpu. Third, electron microscopy revealed that the virions were completely formed but remained tethered to the outer leaflet of the membrane, pointing to the existence of a protein tethering the virions to the cell surface; this mysterious protein at that time was named Tetherin[25].

Comparative microarray analyses eventually identified the mysterious factor to be Bst-2[211]. The IFN-α-mediated upregulation of this restriction factors was a key element for its identification: microarrays analysis were performed in HeLa, HOS, 293T, HT1080 or Jurkat cells treated or not with IFN-α. Less than 10 candidates were endogenously expressed in HeLa cells and upregulated by IFN-α in all the cell lines, but Bst-2 was the candidate that fulfilled all the requirements. Bst-2 is the acronym for bone marrow stromal cell antigen 2 since it was first identified at the cell surface of bone marrow stromal cells[212].

Tetherin and IFN-α

Tetherin is an ISG with anti-HIV-1 activity. Its discovery was based on its IFN-α upregulation and subsequent inhibition of HIV-1 deficient for the Vpu protein[25]. For instance, in 293T and HT1080 cells, HIV-1 deficient for the Vpu protein can replicate as efficiently as the WT virus[25]. However, an IFN-α treatment blocks viral release on these cells[25]. In Jurkat cells and primary lymphocytes, the partial block of viral release is enhanced by IFN-α treatment[25]. Therefore the upregulation of Tetherin by IFN-α was one of the criteria for its selection and subsequent identification. Tetherin levels are upregulated in different degrees, depending on the cell type in which it is expressed: Tetherin was more than 20 times upregulated in 293T cells (mRNA levels) or 2 to 5 times in Jurkat cells[211]. Moreover, Tetherin seems to be completely dependent on IFN-α for its expression and efficient antiviral effect: Tetherin plays a role only when the infected cells are exposed to endogenous IFN-α[213] or treated with IFN-α[124, 213, 214], not like A3G/F, which are expressed at high

basal levels in CD4+ T-cells, which are sufficient for their antiviral effect[23, 149] (see also section *in vivo* studies of Tetherin).

Tetherins' antiviral mechanism

Bst-2/Tetherin is a membrane protein found in the cellular membranes of the endoplasmic reticulum and the plasma membrane, and there, more specifically in the lipid rafts[215] (Fig. 2). It possesses a transmembrane domain on its N-terminus, required for the location of Tetherin in the lipid rafts, and a glycosyl-phosphatidylinositol (GPI) anchor at its C-terminal end. The GPI anchor has the same function as the transmembrane domain since it can be artificially replaced by another transmembrane domain without altering Tetherin's function[216]. The extracellular domain, also named "ectodomain", is located outside of the lipid rafts, accounts for the rest of the protein and contains a coiled-coil domain and several glycosylation sites[215, 217]. This ectodomain is required for microdomain localization and antiviral function[218].

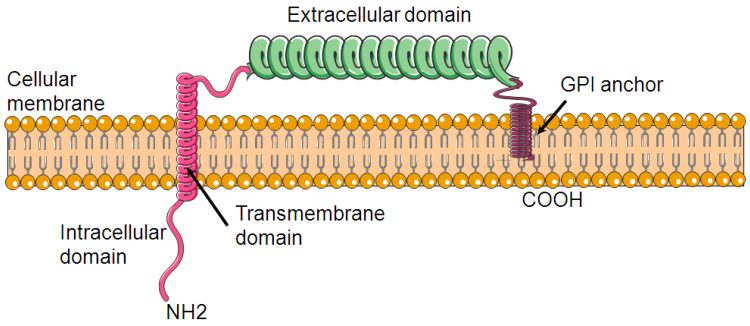

Figure 2. The topology and structure of Tetherin determines its antiviral effect. Tetherin is localized in the cellular membrane and harbors a transmembrane domain in the N-terminal part, an extracellular domain and a GPI anchor in the C-terminal part. In this way, Tetherin is incorporated into the viral particles during budding and tethers them to the cellular membrane.

Cell assays using protease treatment convincingly demonstrated the tethering mechanism of viral particles by Tetherin (see above)[25]. Bieniasz and co-workers[219] generated an artificial Tetherin, which completely differed from the WT sequence but with a similar tridimensional structure, size, post-translational modifications and topology. This artificial Tetherin displayed very potent anti-HIV activity and was insensitive to Vpu degradation. Its

anti-HIV mechanism is thought to interfere with HIV-1 release and cell-cell transmission similar to the natural Tetherin by binding at the same time to the cell surface and to the budding HIV-1 particle [211, 220, 221]. The authors speculated that the artificial Tetherin's mechanism(s) is indicative of the one of the natural Tetherin.

HIV-1 viral production is decreased by Tetherin more than 100 times for Vpu-deficient viruses and only 2 times for HIV-1 WT in 293T cells stably transfected with Tetherin[211]. Tetherin, when overexpressed in 293T cells, also decreases the infectivity of Vpu-deficient strains by interfering with the activation of HIV-1's own protease by an unknown mechanism, leading to an accumulation of pr55Gag and p40Gag precursors[222].

Human Tetherin not only inhibits HIV-1, but also HIV-2 and its related simian Tetherin inhibits SIV, in humans and monkeys, respectively[223-225].

Tetherin neutralization by Vpu

The Vpu protein is unique to HIV-1 and SIV from chimpanzees (SIVcpz)[226]. It is a 16 kDa phosphoprotein of 81 amino acids which harbors two principal domains: the transmembrane N-terminal domain of 27 amino acids, which is hydrophobic, and a cytoplasmic domain of 54 residues[227, 228] (Fig. 2). It is a raft-associated membrane protein, which is generated in the ER, then transported to the Golgi apparatus, where it forms homo-oligomers and is finally translocated to the plasma membranes of the host cells.

Vpu exerts its effect exclusively in the HIV-1 producer cell; Vpu is not incorporated in the newly produced HIV-1 particles, similar to Vif but dissimilar to Vpr and Nef, which are virion-associated proteins[229, 230]. The main function of Vpu is to facilitate the release of viral particles[231] by degrading the CD4 cell surface receptor and Tetherin. CD4, similar to Tetherin, blocks viral particles release by binding of the HIV-1 Envelope.

Vpu co-localizes with and binds Tetherin at the cellular membranes[25, 220]. Overexpression of Vpu in HeLa cells leads to a significant reduction of Tetherin from the cell surface by the proteasomal/lysosomal degradation pathway[220, 232, 233]. The cytoplasmic domain of Vpu possesses a couple of serines (positions 52 and 56) which are constitutively phosphorylated by casein kinase II[234, 235], which allows the recognition and binding of the human protein β-TrCP[234, 235]. Vpu-β-TrCP 2 in concert with an ATPase (p97) polyubiquitinate Tetherin

and CD4 on their cytosolic tail through the E3 ubiquitin ligase, resulting eventually in their proteasomal degradation[26, 236].

As mentioned above, SIV (except for SIVcpz and HIV-2) does not encode Vpu. Instead of Vpu, SIV uses Nef to downregulate monkey Tetherin from the cell surface, without apparent degradation[223, 224]; instead Tetherin is removed from the cell surface by clathrin-mediated endocytosis[237], HIV-2 uses Env to downregulate human Tetherin from the cell surface[225]. Intriguingly, HIV-1 Group P, which originates from gorilla, is unable to counteract human Tetherin even though all the known HIV-1 proteins, including Vpu, Nef or Env, are present[238]. This group of viruses seem less adapted to humans and suggests a protective role of human Tetherin against viral transmission.

In vivo studies of Tetherin

In humans, the antiviral role of Tetherin remains unclear. In a cohort of nine ART-naïve chronically HIV-infected patients, mRNA and protein levels of Tetherin were positively correlated with increased viral load, immune activation and lower CD4+ T-cell counts, suggesting that Tetherin is increased over time as an epiphenomenon of an advanced disease stage[239]. Thus, Tetherin loses its anti-HIV-1 function in advanced HIV-1 disease for whatever reason or its anti-HIV activity is outweighed by other factors promoting viral replication, confounding Tetherins's anti-HIV-1 activity.

However, different results were observed in a study where PBMCs from HIV-infected patients (acute and chronic infection with n=3 and n=10 samples, respectively) were analyzed for the plasma membrane levels of Tetherin, by flow cytometry[213]. There was an increase of Tetherin expression levels during acute HIV-infection and a decrease during the chronic phase, suggesting a role in the viral control from the beginning of the infection. IFN-α treatment of PBMCs from healthy volunteers substantially increased the Tetherin levels, similar to those found in infected patients in acute infection and more interestingly, these Tetherin levels were high enough to decrease viral release of HIV-1 *in vitro*, despite the presence of Vpu[213]. These results show the protective effect of IFN-α during acute infection through Tetherin upregulation and its antiviral effect.

Additionally, exogenous IFN-α treatment lowers HIV RNA copy numbers in HIV-HCV co-infected patients, which were treated for their HCV infection by pegylated IFN-α and

Ribavirin[124]. This decrease in viral load could be due to the antiviral effect of Tetherin, since its levels are increased and since Vpu presented several adaptive mutations as a counteracting strategy against Tetherin. However, only APOBEC3F, APOBEC3G and Tetherin mRNA levels were measured on clinical samples, therefore the effect could also be due to the upregulation of another factor.

Concerning the polymorphisms of Tetherin, there is no data about genetic differences in human patients in the HIV-1-Tetherin context.

In humanized mice, HIV-1 strains deficient in Vpu replicated less efficiently than WT HIV-1[240]. Unlike WT HIV, Vpu-deficient virus did not downregulate CD4 and Tetherin in the spleen of infected mice. These results corroborate the *in vitro* data that Vpu facilitates viral production by degrading CD4 and Tetherin *in vivo*.

Murine and feline Tetherin also have antiviral activities against Moloney murine leukemia virus (MoMLV) and feline immunodeficiency virus (FIV), respectively. In WT mice, the effect of Tetherin was only present when mice were treated with poly I:C (an IFN-α inducer)[214], indicating that basal levels of Tetherin were insufficient to have antiviral activity, and moreover, in Tetherin-knockout mice, this antiviral effect was not induced, indicating that the antiviral effect of IFN-α was specific for Tetherin.

Nevertheless, in mice only one single nucleotide polymorphism (SNP) has been found so far, which induces the expression of higher amounts of murine Tetherin and thus increases its antiviral effect against the Friend retrovirus[241]. Therefore, it indicates that the amount of Tetherin present at the cell surface is an important factor for its antiviral role. In cats, the feline Tetherin decreased particle release of FIV, preventing FIV viremia[242]. Interestingly, FIV is not able to counteract feline Tetherin and FIV spreads by cell-to-cell associated replication[242].

Other functions of Tetherin

Tetherin acts also as an innate immune sensor directly recognizing virions during the budding process, which in turn activates NF-κB in the infected cell and leads to the production of pro-inflammatory cytokines(reviewed in[243]). The activation of NF-κB takes place using the NF-κB canonical pathway, in other words, by interacting with TRAF6 and other cellular

proteins resulting in the phosphorylation and proteasomal degradation of the NF-κB inhibitor IκB(reviewed in[243]). Additionally, the internalization and endocytosis of Tetherin and tethered viral particles for degradation exposes viral elements, such as viral RNA, to different membrane-associated TLRs, like TLR 3, 7 and 8[244].

Tetherin is also present on the cell surface of bone marrow stromal cells and regulates the growth and development of B cells by supporting the microenvironment of the myeloid cell precursors, inducing their proliferation[212, 245-247].

Additionally, Tetherin is upregulated in diverse myeloma cells[248, 249], laryngeal squamous cell carcinoma samples[250] and metastatic breast cancer[251]. Tetherin is the ligand for the immunoglobulin-like transcript 7 (ILT7) in pDCs which subsequent to its binding decreases their immune activation and IFN-α production[252]. Since IFN-α arrests the growth of primary tumors[253], Tetherin may have an immuno-tolerizing effect being induced by cancerous cells.

TRIM5α and Capsid

Discovery of TRIM5α

Tripartite motif-containing 5 alpha (TRIM5α) is up-regulated by IFN type I or II[41]. The HIV inhibitory activity of TRIM5α from rhesus macaques (rhTRIM5α) was discovered when HeLa cells, which were transduced with a cDNA library from rhesus macaque, were infected with HIV-1[254]. rhTRIM5α inhibits HIV-1 approximately fivefold in HeLa cells, by binding the viral capsid[254-256].

In contrast to rhTRIM5a, the human TRIM5α (huTRIM5α) does not restrict HIV-replication. However, it restricts the replication of other retroviruses, like MLV and Foamy Viruses[254, 257, 258].

Another host protein with a similar antiviral activity is CypA. CypA is a peptidyl prolyl *cis/trans* isomerase which catalyzes the isomerization of proline-imidic peptide bonds in oligopeptides and thus accelerates protein folding[259]. However, it has also an antiviral activity by binding capsids of some lentivirus[260]. In owl monkeys and some macaques, retrotransposition of CypA just in front of TRIM5 generated a TRIM-CypA fusion protein (TRIM5-CypA)[261-263] which inhibits HIV-1 to a similar extent as rhTRIM5α.

TRIM5α and IFN-α/β

huTRIM5α is expressed at basal levels in human cell lines, like HeLa, and rhTRIM5α in macaque cells like CMMT and they are upregulated by type I IFN (α and β) or type II IFN (γ)[264]. huTRIM5α is also expressed at basal levels and upregulated by type I IFN in primary human cells such as MDMS, CD4+ T-cells, peripheral blood lymphocytes and DCs[265, 266]. However, this upregulation of huTRIM5α did not result in any HIV-1 inhibition but in the inhibition of N-MLV[264, 267]. Moreover, type I IFN treatment of owl monkey cells (which express TRIM5-CypA)[264] or a rhesus monkey cell line (which express rhTRIM5α)[267] goes along with substantial upregulation of rhTRIM5α and a great increase of the anti-HIV-1 effect of TRIM5-CypA or rhTRIM5α, respectively.

rhTRIM5α antiviral activity

As the name implies, "tripartite motif containing 5 alpha", TRIM5α has three domains, i) a ring finger domain with E3 ubiquitin ligase activity, ii) a B-box domain, and iii) a coiled-coil domain[41] (Fig. 3). The ring finger domain mediates ubiquityation of the viral capsid, which is thought to be important to block infection during capsid uncoating[268]. The B-box and the coiled-coil domain are important for multimerization of TRIM5α and antiviral activity, related to capsid binding[269-273]. In the C-terminal part of the coiled-coil domain is a PRY-SPRY sequence, important for binding of the capsid[257].

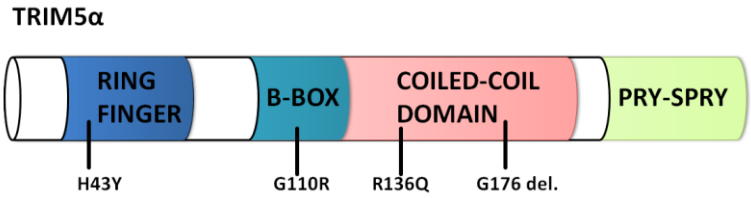

Figure 3. TRIM5α contains three motifs and a C-terminal PRY-SPRY region. TRIM5α is a tripartite motif protein; specifically, it harbors a ring finger, with E3 ubiquitin ligase activity, a B-box, and a coiled-coil domain. In the C-terminal part of the coiled-coil domain of TRIM5α, there is a PRY-SPRY sequence. SNPs modifying the antiviral activity of TRIM5α are depicted, such as the SNP H43Y in the ring finger, the G110R in the B-Box and the R136Q and G176 deletion in the coiled-coil domain.

The molecular mechanism of rhTRIM5α's anti-HIV activity is not entirely clear: one model proposes that binding of the viral capsid by the PRY-SPRY induces capsid disruption and thus impedes the subsequent steps of the viral life cycle[274]. Another model suggests that the ring-finger domain viral capsid degradation by a proteasome-dependent mechanism[275]. Treatment with proteasome inhibitors or mutations in the ring finger, however, do not abolish the restrictive phenotype when HeLa cells encoding for rhTRIM5α were infected with HIV-1[276], indicating that proteasome-independent mechanisms must also be operative in TRIM5α's anti-HIV activity.

As mentioned above, huTRIM5α has no anti-HIV activity[254]. A non-synonymous mutation in the PRY-SPRY domain (R332P), however, transforms huTRIM5α into a potent HIV-1 restriction factor[277, 278]. This non-synonymous mutation was found by constructing chimeras between human and rhesus TRIM5α[277]. It remains unanswered whether there was a loss of function in humans and/or a gain in rhesus monkeys, respectively. In any case, the specific amino acid rendering huTRIM5α anti-HIV active has not been reported so far in HIV-1 elite controllers.

***In vivo* studies of TRIM5α**

Although huTRIM5α has no obvious anti-HIV activity, some SNPs in different ethnicities appear to affect HIV-1 transmission rate. Comparing Japanese and Indian cohorts of HIV-infected individuals with healthy volunteers, the SNP G110R was more frequent in HIV-infected individuals whereas the SNP G176del was more frequent in the healthy volunteers; both mutations are located in the B-Box and the coiled-coil domain of TRIM5α, respectively[279]. *In vitro* tests showed that G110R decreases the anti-HIV-1 activity of a human/AGM chimeric TRIM5α (which possessed the SPRY domain of AGM TRIM5α), whereas the G176 deletion increased the chimeric TRIM5α activity against HIV-1. The ultimate mechanism underlying these effects is unknown.

Two other SNPs, H43Y (located in the ring finger) and R136Q (located in the coiled-coil domain), increase susceptibility to HIV-1[280, 281] or are enriched in HIV-resistant individuals, which are highly exposed to HIV-1[281, 282], respectively. The protective effect of R136Q was only observed against X4-tropic viruses, which infect naïve CD4+ T-cells, and with moderate-to-high viral loads[281]. The mechanistic explanation for this protective effect

would be that naïve CD4+ T-cells express higher levels of TRIM5α than memory CD4+ T-cells, which are infected by R5-tropic viruses. *In vitro*, R136Q and H43Y variants were tested for their capability to restrict HIV-1[283]. Although the R136Q mutant restricted different capsid variants better than the H43Y mutant, it did not restrict HIV-1 better than WT TRIM5α. However, for some capsid variants tested there were different degrees of susceptibility to restriction by TRIM5α, indicating a selective pressure on the viral capsid.

Together, these studies indicate that huTRIM5α has very limited anti-HIV effect but rather a potent antiviral effect against other non-human retroviruses, impeding zoonotic viral infections.

Other functions of TRIM5α

Besides its anti-HIV effect, TRIM5α acts also as a pathogen recognition receptor (PRR) of the retrovirus capsid lattice, by triggering MAPK and NF-κB-dependent genes, leading to the activation of TAK1 and inflammatory transcription[284].

SAMHD1 and Vpx

Discovery of SAMHD1

Monocytes are rather resistant to HIV-1, while HeLa cells expressing CD4 are highly permissive. Heterokaryons made by monocytes and Hela cells display the same non-permissiveness as monocytes, pointing to a dominant restriction factor present in monocytes but not in HeLa cells[285]. However, Vpx provided in *trans* renders cells of myeloid origin highly permissive to HIV-1[286-288]. The Vpx protein (acronym for viral protein X) is an accessory protein encoded by HIV-2, SIVsm (sm: sootey mangabeys) or SIVmac (mac: rhesus macaques), which is incorporated into newly generated viral particles[289]. It is a small 112 amino acid protein[290] migrating at 16 kDa[291]. If HIV-1 was engineered to incorporate Vpx into the virions, cells of myeloid origin got highly permissive[292]. Thus, Vpx appears to counteract this restriction factor in cells of myeloid origin.

Benkirane and co-workers identified SAMHD1 (sterile alpha motif and HD domain protein 1) as the restriction factor which is neutralized by Vpx using mass spectrometry and co-immunoprecipitation studies, by comparing parental THP-1 cells with THP-1 cells genetically complemented by Vpx[293]. SAMHD1 is present in monocytes and MDDCs and absent in

HIV-1 permissive cell lines. Monocytic cell lines silenced for SAMHD1, e.g., THP-1, are 16 times more permissive to HIV-1 infection than parental cells[294].

SAMHD1 and IFN-α

Originally, SAMHD1 was previously identified as a factor induced by IFN type II in macrophages and dendritic cells in the context of differentially expressed genes in macrophages and DCs[37, 295]. It was thought that SAMHD1 is only upregulated by IFN type II, but it is also upregulated by IFN-α in MDMS[296] and by other cytokines such as IL-12 and IL-18[297]. Of note, in MDMS treated with an anti-IFN-γ–neutralizing Ab, the effect of IL-12 and IL-18 was maintained[297]. Similarly to A3G/F (see above), SAMHD1 is highly expressed at basal levels in immune cells such as primary MDDCs or CD4+ T-cells, but is not upregulated at the protein level by IFN-α treatment[38, 298]. However, SAMHD1 protein levels can be significantly upregulated in cell lines such as 293T and HeLa.

SAMHD1 antiviral mechanism

SAMHD1 is a dGTP-stimulated triphosphohydrolase, i.e., it hydrolyses the deoxynucleoside triphosphates (dNTPs) into deoxynucleoside and inorganic triphosphate[299, 300]. This hydrolysis results in a depletion of the dNTP pool of the cell[88]. Reverse transcription of the HIV-1 single stranded RNA is dependent upon dNTP and thus depletion of the dNTP pool inhibits viral cDNA synthesis[299].

SAMHD1 is phosphorylated in the position T592 in dividing cells, whereas in quiescent cells this phosphorylation is absent[301]. Phosphorylation results in the loss of SAMHD1's hydrolase activity and thus in the bigger part of its antiviral activity. IFN type I treatment inhibits this phosphorylation on Thr592 residue without modifying the expression levels of SAMHD1, increasing the antiviral effect of SAMHD1 on activated CD4+ T cells, MDMS and MDDCs. This might explain why the antiviral effect of IFN-α takes place without an increase of the expression levels of SAMHD1 in different immune cells.

Importantly, SAMHD1, as opposed to the previously mentioned restriction factors, is able to counteract WT HIV-1. Its expression is limited to the myeloid lineage (macrophages, monocytes, DCs)[286] and quiescent CD4+ T cells[89, 302]. In myeloid cells, SAMHD1 is

thought to impede a prompt immune response by blocking HIV-1 replication at early stages of infections and subsequently impeding IFN-α production.

SAMHD1 not only restricts HIV-1 and other retroviruses, but also DNA viruses, like vaccinia and herpes simplex virus type 1 (HSV-1), by the same mechanism[303].

SAMHD1 neutralization by Vpx

Vpx promotes the binding of SAMHD1 to the CRL4 (DCAF1-Vpx) E3 ubiquitin ligase complex by interacting with the DCAF1 substrate receptor subunit, leading to SAMHD1's proteasomal degradation[90]. SAMHD1 is mainly a nuclear protein, and it harbors a nuclear localization signal (amino acids 11KRPR14) (Fig. 4). The Vpx-induced degradation takes place in the nucleus whereas the cytoplasmic fraction of SAMHD1 is resistant to Vpx and is thus able to deploy its activity[304, 305]. Furthermore, the binding of Vpx to SAMHD1 in the nucleus, prior to degradation, inhibits its catalytic activity[306]. Counteracting the intrinsic innate immune response appears to be a common strategy of the HIV-1 accessory proteins, including the HIV-1 Vif, Vpu and the HIV-2 Vpx. Counteracting SAMHD1 by Vpx provided in *trans* results in efficient HIV-1 infection of DCs or monocytes with ensuing high production of IFN-α and activation of the DCs[91]. Currently, it is speculated that HIV-2 is less pathogenic than HIV-1 due to its ability to productively infect cells of myeloid origin and in particular DCs which induce a prominent IFN-α response – the inability of HIV-1 to productively infect these cells will not lead to this first wave of IFN-α production.

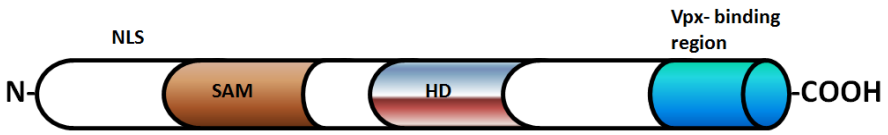

Figure 4. SAMHD1 has two different domains for its antiviral activity. The Sterile Alpha Motif (SAM) and the HD domain are depicted. Additionally, there is a region where Vpx binds SAMHD1 for its degradation. The nuclear localization signal (NLS) required for the nuclear localization of SAMHD1 is also depicted.

In vivo studies of SAMHD1

The SNP rs1291142 was associated with lower mRNA levels of SAMHD1[307]. However, no association was observed for this SNP in cohorts of European and African-American HIV-positive patients with distinct HIV-1 progression rates, i.e. elite controllers vs. progressors.

Loss of function mutations in the SAMHD1 gene are responsible for the Aicardi-Goutières syndrome[308, 309] or familial chilblain lupus[310]. Aicardi-Goutières syndrome is a neurodegenerative disease which is characterized by a high level of IFN-α in the cerebrospinal fluid and a progressive encephalopathy (reviewed in[311]). The symptoms resemble those of viral infections and the disorder is due to a lack of suitable processing of cellular nucleic acid debris, triggering permanently innate and acquired immune responses[312]. These mutations alter the localization of SAMHD1, leading to its accumulation in the cytoplasm. As mentioned above, SAMHD1 is mainly present in the nucleus. Not surprisingly, without a functional SAMHD1, PBMCs from Aicardi-Goutières syndrome patients support HIV-1 replication without any exogenous activation and moreover, the monocytes of these patients are highly permissive to HIV-1[296].

Other IFN-α/β/γ-stimulated restriction factors

Several other anti-HIV-1 restriction factors, which are stimulated by IFN-α/β/γ, have been identified so far and are therefore mentioned here. However, their potency is less than that one of those reviewed above and their antiviral mechanisms remain largely unknown. Additionally, there are no publications studying the anti-HIV effect of these factors depending on IFN-α production.

TRIM22

TRIM22 (another tripartite motif protein) was identified in a screening with a cDNA library from the lymphoblastoid Daudi cell line treated with IFN-α/β and was initially named Staf-50 for Stimulated Trans-Acting Factor of 50 kDa[313]. Similarly to TRIM5α, TRIM22 is also upregulated upon IFN type I or II treatment[313]. For instance, in monocyte-derived macrophages, this factor is upregulated upon IFN-α treatment[314]. An overexpression of TRIM22 efficiently inhibits HIV-1 replication by blockingHIV-1 transcription[314], directly at the LTR promoter region[315]. For this, it has been speculated that TRIM22 interferes with Tat- and NF-κB-independent LTR-driven transcription. Additionally, it was also reported that

TRIM22 could restrict HIV-1 replication by altering Gag trafficking[43]. In associative *in vivo* clinical studies, TRIM22 expression was positively correlated with endogenous levels of IFN-α in HIV-1-infected patients during acute infection, among other factors[316]. Moreover, there was a negative correlation between TRIM22 and viral load and a positive correlation with CD4+ T-cell counts, indicating a protective role of TRIM22.

RNase L and 2-5A

RNase L and 2, 5'-linked oligoadenylate (2-5A) were simultaneously discovered in Ehrlich ascites tumor cells (EAT), which were treated with IFN (not specified which type of IFN) and the enzymes were purified by different biochemical procedures. RNase L and 2-5A were highly upregulated in these cells and their protein levels were abundant enough to be purified[317]. Later on, it was discovered that HIV-1 can be inhibited by an overexpression of RNase L[35]. This RNAse is dependent on 2-5A, which is also inducible by IFN-α. RNase L acts by degrading viral and cellular RNAs. However, HIV-1 appears to induce an RNase L inhibitor (RLI) resulting in productive HIV-1 replication.

PKR

The Protein kinase RNA activated (PKR) was discovered for its ability to inhibit protein synthesis in rabbit cell's lysates exposed to double-stranded RNA[318]. Later on, looking for partners of the HIV-1 Tat protein, with GST fusion chromatography, it was shown that Tat binds the PKR protein. PKR is another IFN-α-stimulated protein that can inhibit viral replication[319]. This kinase can be activated by different means, such as sensing dsRNA, pro-inflammatory stimuli, or cytokines[34]. Activation and over-expression of this protein highly blocks HIV-1 expression and replication, by inhibiting protein translation. However, HIV-1 has developed different strategies to counteract this barrier. Among them, the viral Tat protein[34] or the expression of cellular dsRNA binding proteins (e.g. trans-activation response RNA-binding protein and ADAR1), inhibit PKR activation and thus its antiviral activity[320-322].

ISG15

ISG15 was discovered in experiments where the researchers were looking for ISGs[323]. This was done by loading protein gels with cell lysates of cells stimulated or not with IFN type I. Subsequently, ISG15 was tested against HIV-1 and it was observed that ISG15 inhibits viral

budding and release, by impeding Gag and Tsg101 ubiquitination and their interaction, necessary for viral release[324]. Tsg101 is a protein implicated in endosomal sorting[325]. Moreover, ISG15 inhibits viral budding by decreasing Vps4 ATPase activity through targeting the CHMP5-LIP5 complex[326]. Of note, the cellular ATPase Vps4 is involved in endosomal sorting[327].

IFITM protein family

The interferon-induced transmembrane (IFITM) proteins were identified as ISGs with anti-HIV activity by using short hairpin RNA (shRNA) against ISGs present in SupT1 cells[328]. IFITM protein members 1, 2 and 3 are induced by IFN type I. These proteins restrict HIV-1 at the level of viral entry, at the endocytosis pathway (IFITM 2 and 3), and also by decreasing Gag expression (IFITM 1, 2 and 3). Moreover, the C-terminal part of IFITM proteins is responsible for the viral restriction.

HERC5

HECT domain and RCC1-like domain-containing protein 5 (HERC5) was discovered by a cDNA screening of endothelial cells stimulated with LPS or TNF-α[329]. Subsequently, in another study, the authors tested IFN-β-induced factors previously identified for their anti-HIV activity. Since HERC5 is also IFN-β-inducible, it was identified as a new restriction factor among the upregulated proteins[29]. HERC5 inhibits HIV-1 replication at a late stage of particle production, specifically, by arresting Gag particles at the assembly site on the plasma membrane. It was recently documented that HERC5 blocks HIV-1 replication by inhibiting the nuclear export of Rev/RRE-dependent RNA by altering Rev localization[330].

CNP

2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) was discovered by screening for ~50 ISGs transduced in 293T together with an HIV-based lentiviral[27]. CNP was responsible for a decreased viral protein production. CNP is a membrane-associated protein, which counteracts HIV-1 by binding to the structural retroviral protein Gag[27]. In this way, it blocks viral particle assembly once both Gag and the viral RNA have associated at the plasma membrane. Sensitivity or resistance to CNP is determined by a single codon (E/K40) in the matrix domain of Gag. Finally, CNP presents a spectrum of interspecies variation in antiviral activity.

SLFN 11

Schlafen 11 (SLFN 11) belongs to the protein family Schlafen, which is involved in thymocyte maturation and is expressed in lymphoid cells[331]. Interestingly, 293 cells produced less HIV-based lentiviral particles as compared to 293T cells[39]. The researchers discovered that SLFN 11 is expressed in 293 cells but not in 293T cells and thus was postulated to be responsible for the phenotype observed. Additionally, other restriction factors such as APOBEC3G or ISG15 were discarded since there was no difference in their expression in the two cell lines 293 and 293T. SLFN 11 is highly upregulated in murine macrophages and human fibroblasts by poly-IC or IFN-α/β treatment. SLFN 11 acts at the level of production of viral proteins in a "codon-usage-dependent manner"[39]. To do so, SLFN 11 binds transfer RNA (tRNA) and inhibits changes in the tRNA pool that HIV would otherwise induce. It is important to mention that the inhibitory activity of this factor is only against viral proteins but not cellular proteins. As mentioned above, in the previous section "IFN-α: good or bad in HIV-1 infection", this factor is significantly upregulated in HIV-1 elite controllers.

MX2

Myxovirus resistance 2 (MX2) is an interferon-induced protein which was discovered by comparing the transcription profiles of monocytic cell lines (THP-1), primary CD4+ T-cells and several CD4+ T-cells lines (HT1080 and U87-MG cells), which present an inhibitory phenotype against HIV-1 when stimulated with IFN-α, as compared to other cell lines which do not exhibit this restrictive effect such as Jurkat, CEM, CEM-SS, K562 and U937 cells[32, 33]. MX2 inhibits early steps of the HIV-1 replication cycle, in particular HIV-1 nuclear import or integration. The Capsid region of the viral Gag protein is responsible for the susceptibility of HIV-1 to this factor, as shown by mutations in the Capsid, which abolished the antiviral effect. It is not known yet how HIV-1 manages to escape this factor, but one can speculate that HIV-1 mutates its capsid when this factor is upregulated upon IFN-α stimulation or exogenous IFN-α treatment.

Conclusions and perspectives

Since the unrestricted activity of APOBEC3 in Vif-deficient HIV-1 infections results in abortive HIV-1 infection, interfering with Vif neutralization of APOBEC3 enzymes may

provide a very attractive therapeutic strategy. Similarly, for Tetherin, one may envision to inhibit either the Vpu/Tetherin interaction or the degradation of Tetherin by Vpu. Or for TRIM5α, a drug that is able to mimic the molecular effects of TRIM5α could be designed, by binding the viral capsid and sending it for its proteasomal degradation.

Alternatively, given that the mutation R332P renders huTRIM5α completely effective against HIV-1 and the SNP R136Q and the G174 deletion seem to increase HIV-resistance, one can think about a gene therapy approach based on a modified huTRIM5α harboring these three mutations. The R136Q mutation would protect the naive CD4+ T-cells against infection by X4-tropic viruses, which would decrease the chances of developing AIDS. However, this huTRIM5α harboring these three mutations has not been tested *in vitro* against HIV-1 yet.

Similarly, since SAMHD1 is able to counteract HIV-1 WT in different myeloid and lymphoid cells, therapies targeting the upregulation of SAMHD1 should be considered, in order to develop alternative anti-HIV-1 treatments. Additionally, learning about how the restriction factor works and how HIV-1 is able to counteract it or escape its action can provide the researchers with new ideas how to develop new antiviral drugs and new vaccine and cure strategies.

Although called "accessory proteins", these viral factors can be essential for the virus through inhibition of several restriction factors, as shown for Vif, Vpu and Vpx against APOBEC3G/F, Tetherin and SAMHD1 respectively. It is likely that more restriction factors exist which might be counteracted by the accessory proteins. There might also be other IFN-stimulated restriction factors, which HIV-1 is able to escape from. It was recently shown that a chimeric HIV-1/SIV_{mac}, which is able to counteract the known restriction factors APOBEC3, Tetherin, SAMHD1 and TRIM5α, is highly inhibited upon IFN-stimulation, indicating existence of more restriction factors upregulated by IFN-α[134, 332].

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1.3 Modelling HIV infection and therapies in humanized mice

Since HIV-1 only replicates in humans and chimpanzees, it is necessary to develop a murine model that supports HIV-1 replication for *in vivo* studies. Different immunodeficient murine strains have been created in order to provide a human immune system that allows HIV-1 replication, called humanized mice. In order to describe the different humanized mice used today for HIV-1 *in vivo* studies, the next review was written.

Authorship contributions

I wrote the review with Roberto Speck and Marc Nischang and I draw the figure present. Annette Audigé and Ramesh Akina corrected the review.

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Modelling HIV infection and therapies in humanised mice

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Summary

The human immunodeficiency virus (HIV) type-1 is a human-specific virus. The lack of a widely available small-animal model has seriously hampered HIV research. In 2004, a new humanised mouse model was reported. It was based on the intrahepatic injection of human CD34+ cord blood cells into newborn, highly immunodeficient mice. These mice develop a lymphoid system of human origin and are highly susceptible to HIV infection and showed disseminated infection, persistent viraemia and characteristic helper CD4+ T-cell loss. Here, we will briefly review the various existing humanised mouse models and highlight their value to the study of HIV infection.

Key words: *HIV-1 infection; HIV pathogenesis; humanised mice; BLT mice; SCID; human CD34+ cells; gene therapy; CCR5; antiretroviral treatment; microbicides; HIV pre-exposure prophylaxis (PrEP)*

The HIV situation globally

The UNAIDS report on the Global AIDS Epidemic 2010 optimistically announced that the HIV pandemic had peaked in the preceding two years. Nevertheless, the numbers are shocking: an estimated 33.3 million humans – 0.8% of all adults 15–49 years old – are infected, and more than 1.8 million people died in 2009 (http://www.unaids.org/globalreport/Global_report.htm).
More hopefully, the number of people newly infected with HIV declined by nearly one-fifth over the last decade (1999, 3.1 million; 2009, 2.6 million). This decline is based on more widely applied "safer sexual practices" and reductions in mother-child transmission. The UNAIDS vision is Zero New Infections, Zero Discrimination, Zero HIV-associated Deaths. The goal is to halt and reverse the spread of HIV.

A human-specific virus: a challenge for *in vivo* studies

HIV specifically infects human cells. Even cells from chimpanzee, a very close relative of humans, are only somewhat permissive to HIV infection [1]. Human host factors are critical for the virus throughout its entire replication cycle (fig. 1). For example, to enter a cell and begin its replication cycle, HIV engages a receptor complex of CD4 and a chemokine receptor, either CCR5 or CXCR4 [2, 3]. However, expressing human CD4 on murine cells does not make them permissive to HIV. Other human-specific factors, such as the human chemokine receptors, are needed.
Over the last three decades, human transgenes essential for HIV replication were expressed together in rodent cells, but the cells were still not permissive [4]. Furthermore, human transgenes were expressed in rodents in an attempt to generate HIV small-animal models. These models confirmed the human-specific nature of HIV and the *in vitro* data. No replication was observed in mice expressing human CD4 [5], CD4 and CCR5 [6] and Cyclin CDK9 [7], and rats transgenic for human CD4 and CCR5 replicated HIV only at very low levels for limited times [8, 9].
Other *in vivo* studies attempted to create models based on creating chimeric HIV strains. This approach relies on engineering a distinct HIV gene in a species-specific retrovirus, which despite the HIV transgene, replicates vigorously in the original species (e.g., simian-immunodeficiency virus [SIV] engineered to express the HIV envelope [SHIV]) [10]. The use of SHIV in monkeys allowed key questions about immune responses to vaccine constructs expressing various HIV gene encoded proteins to be addressed [10]. However, use of monkeys as animal models is restricted to specific questions with a narrow focus and cannot recapitulate the overall complexity of HIV, since the biological properties between SHIV, SIV and HIV are quite distinct.
Finally, HIV-encoded gene products were expressed entirely as transgenes in mice [11–15]. These studies provided insight into the pathogenic potential of HIV gene products. However, they were expressed universally at high levels, and it is difficult to assess the significance of the resulting data since the dynamic nature of true HIV replication is lacking.

The requirements for a mouse model to study HIV infection

Faithfully modelling any human disease in an animal is difficult. Does the model replicate enough key features of the disease to allow us to conduct experiments?

Many of the key features of HIV infection are known. The main route of HIV transmission is vaginal or rectal intercourse. In acute HIV infection, a massive productive infection causes cell death in the lymphatic system, most prominently in the gastrointestinal tract. About 3–5 weeks after acute HIV infection, the levels of HIV RNA decline and the specific anti-HIV CD8+ T-cell response begins. Unlike in acute infection, fewer than 1% of CD4+ T cells are productively infected in the chronic phase [16], a number that cannot fully explain the progressive immunodeficiency. Poorly understood bystander effects seem to contribute to the overall cell loss [17], and sustained immune activation triggers it [18]. Combined antiretroviral treatment (cART) has been very successful in suppressing HIV RNA levels to below the limit of detection in about 90% of treated patients [19] and has resulted in a marked reduction of morbidity and mortality [20]. However, cART does not cure HIV. A small portion of HIV remains silent in long-lived cells, such as the quiescent memory CD4+ T-cells [21]; these cells form a latent reservoir of HIV. Besides finding simpler and more efficient treatment strategies, major efforts are now aimed at eradicating the latently infected cells to eventual cure HIV, and to develop novel gene therapy approaches and vaccination strategies. Other efforts are focused on orally administered pre-exposure prophylactic measures using anti-retroviral drugs and finding effective topical microbicides that can prevent sexual transmission.

Thus, the requirements for a HIV mouse model include the following:

– Permissiveness to replication-competent HIV with distinct co-receptor usage (i.e., CCR5- or CXCR4-tropic HIV strains), resulting in high-level viraemia, systemic viral dissemination and histopathology reminiscent of HIV disease in humans.
– Supporting long-term chronic infection, allowing monitoring of HIV infection over time.
– Susceptibility to natural transmission modes of HIV, including vaginal and rectal routes.
– Displaying gradual depletion of CD4+ T-cell numbers during HIV infection.
– Activation of the immune system to lead to HIV-specific immune responses.
– Establishment and maintenance of an HIV latent reservoir.
– Allow development and testing of anti-HIV therapeutic and prevention strategies.

Humanised mice in general

The generation of humanised mice involves either the expression of human transgenes or the transplantation of human tissue into immunodeficient mice. However, as mentioned above, even constitutive expression of multiple human transgenes has not rendered mice fully permissive to HIV infection.

The human-PBL-SCID and foetal thy/liv SCID mouse model

Transplantation of human tissue into immunodeficient mice without rejection was first reported in the early 1980s. This became possible with the identification of a spontaneous mutation of the *Prkdc* gene in mice, which results in the complete lack of T and B cells and consequently in severe combined immunodeficiency (C.B.-17 SCID/SCID [SCID]; descriptions of the various mouse strains, see box) [22]. The *Prkdc* gene encodes for the catalytic subunit of a DNA-dependent protein kinase that is needed for V(D)J recombination in developing T and B lymphocytes. The two early humanised (Hu) mouse models were the foetal thymus/liver (thy/liv) SCID-hu mouse [23, 24] and the hu-PBL-SCID (PBL, peripheral blood leucocytes) mouse [25]. The foetal thy/liv SCID-hu mouse model is based on surgical placement of foetal thymus/liver tissue under the renal capsule. At 4–6 months post-implantation, foetal thymus/liver tissue forms a conjoint organoid that resembles human thymus and sustains T-cell lymphopoiesis for over a year [23]. The system is susceptible to HIV infection, but in the absence of robust peripheral human leukocyte reconstitution, samplings to analyse the infected human cells are mainly restricted to the engrafted conjoint organoid. Also there is no multilineage human haematopoiesis in this model (table 1).

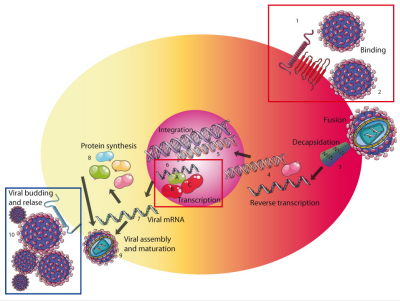

Figure 1

HIV-1 needs critical host factors for efficient replication. HIV binds to the HIV receptor complex of the human CD4 cell-surface molecule and a co-receptor, either CCR5 or CXCR4, via the HIV envelope glycoprotein 120 (HIV env gp120). After conformational changes in the HIV env gp41, viral host cell membrane fusion occurs (2). The next steps are the decapsidation (3) and release of the HIV RNA from the virus particle. Reverse transcription generates a viral complementary DNA (cDNA) based on the viral RNA template and using HIV's own reverse transcriptase (4). Once the cDNA is generated, the preintegration complex (PIC) is assembled, nuclear trafficking and integration of the viral cDNA into the host genomic DNA follow (5). Efficient transcription and elongation require formation of P-TEFb (positive transcription elongation factor b) consisting of Tat (6A), human cyclin-dependent kinase 9 (CDK-9) (6B) and cyclin T1 (6C), which binds to the nascent HIV transcripts. Fully or partially spliced HIV mRNA (7) is used to translate viral proteins. Unspliced HIV RNA is packaged into newly generated virions. Assembly of HIV proteins and RNA and budding takes place at the cellular membrane (9). HIV release is inhibited by murine tetherin at the cellular membrane because murine tetherin is insensitive to the viral protein Vpu, which inhibits human tetherin by directing its proteasomal degradation (blue frame). Human host factors critical for HIV replication are CD4, CCR5, CXCR4 and cyclin T1 (red frame). Additional human specific factors probably exist.

The hu-PBL-SCID mouse model is based on the intraperitoneal injection of human PBL [25] and is susceptible to HIV infection [26]. However, within days, human PBL injected into mice react against the murine disparity with a vigorous activation: their proliferation rate increases, and the CCR5 chemokine receptor and HLA-DR are upregulated [27–29], resulting in xeno-reactive T-cells [30]. Mice with significant blood T-lymphocyte chimerism suffer from high levels of graft-versus-host disease (GVHD) and mortality. Mice with no or transient T-cell chimerism have a low incidence [31]. Use of this model is limited mostly by the lack of *de novo* development of continuously differentiating human cells, activation status of the xenoreactive T cells and the GVHD (table 1).

New approaches for generating humanised mice

In 2004, a novel humanised mouse model was reported. It was based on transplanting human CD34+ haematopoietic progenitor cells (CD34+ cells) directly into the liver of newborn immunodeficient mice (Rag2$^{-/-}$ $\gamma_c^{-/-}$) [32]. By 10 weeks after transplantation, the mice develop a lymphoid system of human origin with T cells, B cells, NK cells, monocytes and dendritic cells. Notably, the T cells display a pattern of naive and memory cells and a Vβ repertoire similar to that of humans. The mouse mounts a specific antibody response against model antigens, such as pneumococcal and tetanus toxoid antigens, but the response is much weaker than that in humans (table 1).

This model is a significant step toward humanisation. Importantly, the mice lack the γ_c chain, which results in even more drastic immunodeficiency as compared with SCID mice. The γ_c chain is an essential component of the IL-2, -4, -7, -9, - 15 and -21 receptors. Its absence severely compromises the development of immune cells, including NK-cell development, and thus their rejection potential against transplanted xenogeneic tissue. It also makes the mice less susceptible to lymphoma development. Indeed, the NOD-SCID IL-2Rγ-null mice are much more useful than the NOD-SCID mice for transplanting human tissue [33]. Notably, NOD-SCID IL-2Rγ-null mice show a similar degree of immunodeficiency as Rag1 or 2-/- γ_c -/- knock-out mice; they have been developed by crossing of SCID mice with non-obese diabetic (NOD) mice and mice deficient in the gamma c (γ_c) chain of the IL-2 receptor [34].

Table 1: Compilation of the various humanised mouse models.

Humanised mouse model	Engraftment	Cellular composition in reconstituted hu mice	Supports HIV infection with	Advantages	Disadvantages
hu-PBL SCID [25]	N/A	• T and B cells	• CCR5 and CXCR4-tropic strains[§] [26, 28, 62]	• Easy to generate (i.e. good access to PBLs) • Can immediately be used after transfer of PBLs	• No multilineage haematopoiesis • Limited time frame for experiments [62] • Strong activation of T-cells [27, 28] • Emergence of xeno-reactive T-cells (GvHD) [30]
Thy/Liv SCID hu [23]	N/A	• T cells • Single positive double positive and double negative Thymocytes	• CCR5-* and CXCR4-tropic strains [24, 58, 59]	• Organoid of foetal thymus/liver tissue with sustained T-cell lymphopoiesis • Valuable to study certain pathogenic aspects (see text)	• Surgical skills needed • Human foetal tissue needed • No multilineage haematopoiesis • Sampling mainly restricted to the organoid since lack of solid peripheral reconstitution • Lack of CCR5 expression on intrathymic T progenitor cells
Rag2-/- γc -/-[32]	++	• T and B cells • Monocytes • Macrophages • NK cells • DCs	• CCR5- and CXCR4-tropic strains [68, 69, 71]	• Long-term multilineage haematopoiesis • Specific antibody response to recall antigens [32] • Suited to study HIV pathogenesis [118, 131, 132], HIV latency [90], gene therapy and novel anti-HIV treatment approaches [90]	• Delay between transplantation of human CD34+ cells and development of lymphoid system of ~15 weeks.
NOG [38] or NSG [33]	+++	• T and B cells • Monocytes • Macrophage • NK cells • DCs	• CCR5- and CXCR4-tropic strains [40, 70, 131, 132]	• Higher reconstitution levels as compared to Rag mice [39, 41] • Suited for studying HIV pathogenesis [119, 123], HIV treatment [95, 98] and latency and gene therapy approaches [102, 107]	• Sensitive to irradiation
NOD/SCID ¶ -hu BLT [42] And NOD/SCID γc -/- (NSG) BLT [133]	+++	• T and B cells • Monocytes • Macrophages • NK cells • DCs	• CCR5- and CXCR4-tropic strains [43, 77, 80, 88, 108, 115]	• Generation of adaptive immune responses [115] • Suited for studying HIV pathogenesis [43, 115], anti-HIV treatment [77, 80, 108], HIV latency [88] as well as novel gene therapies	• Two step procedure for generating BLT mice • Surgical skills needed • Human foetal tissue needed

N/A = not applicable
\+ = good engraftment
++ = high engraftment
+++ = very high engraftment
[§] Infection using CXCR4 tropic HIV-1 strains only successful shortly after transfer of human PBLs.
* Controversial data concerning the permissiveness of foetal thy/liv SCID mice to infection with CCR5 tropic HIV-1 strains.
¶ For simplicity reasons, we put together these two subtly different models; in fact, BLT mouse using NSG background show a superior engraftment as compared to NOD/SCID BLT mice.

Modifications for improving the engraftment of human haemato-lymphopoietic tissue have been investigated, including the use of human foetal liver derived CD34+ cells, cultivating the CD34+ cells with a cytokine cocktail before transplantation [35], pre-conditioning the mice with busulfan instead of irradiation [36, 37], the use of different mouse strains, such as NOD/shi-scid/$\gamma_c$null (NOG) [38] or NOD/SCID/γ_c -/- (NSG) mice [39], the transplantation of CD34+ cells intravenously or into the bone marrow or the transplantation of CD34+ cells at older age of the mice [39]. (NSG and NOG mice are nearly identical except for the modification of the γ_c chain receptor; in both strains, triggering through the γ_c chain receptor is disabled: in NSG mice the receptor is completely knocked down, and in NOG mice the intracytoplasmic tail is truncated). NOG mice are especially vulnerable to developing lymphomas after irradiation; however, they yield similar engraftment results even when not irradiated [40]. Very importantly, the lifespans of humanised mice, except of irradiated NOG mice, appear to be similar to those of wildtype mice; the mice eventually die due to infirmity. NSG mice transplanted at birth with haematopoietic progenitor cells either from human foetal liver or from human cord blood gave the better engraftment than the Balb/c-Rag1-/- and C.B-17-scid/bg mice [41]. Similar data have been reported by Brehm et al. [39].

The BLT mouse deserves special mention [42]. BLT is an acronym for bone marrow liver thymic. In this model, foetal liver/thymus is placed under the renal capsule in 6–8-week-old immunodeficient mice as with standard SCID-hu mice. However, after 3 weeks, the mice are sublethally irradiated, and autologous human CD34+ cells are transplanted into the mice. These cells home to the bone marrow and also migrate to the scaffold generated by the initial transplantation of the human foetal liver/thymus tissue. In the BLT mice, engraftment of human lymphoid tissue is highly efficient, even to the gastro-intestinal tract [43]. The innate and adaptive immune responses appear to be generally more complete in the BLT mice than in humanised mice generated by transplanting human CD34+ cells alone [42]: BLT mice generate a human MHC-restricted T-cell response to Epstein Barr virus (EBV) and activated Vβ2-TCR+ T-cells when dendritic cells present the superantigen toxic shock syndrome toxin 1 (TSST-1). Notably, TSST-1 specifically activates and induces the TCR Vβ2+ cells to proliferate. Generating an adaptive immune response is facilitated by educating the human T-cells in an autologous thymic microenvironment. This is not the case in the other humanised mouse models, which have xenogeneic mouse thymic environments. To overcome this limitation, immunodeficient mice were generated expressing the human HLA class I genes [44]. Here, mice transplanted with HLA-matched cord blood cells supported the *in vivo* differentiation of functionally mature human cytotoxic lymphocytes associated with a wide spectrum of functional human T-cell subsets. The mice mounted an EBV-specific immune response upon challenge as quantified by tetramer staining and enzyme-linked immunospot (ELISPOT) assay.

Thus, introducing human HLA-class I transgenes significantly improved the humanisation of the mice. Similarly, a new report demonstrated expression of class II (HLA-DR4) in NOD- Rag1 -/- /γ_c -/- mice and consequent improvement in T- and B-cell development and function [45]. Additional human transgenes critical for haematopoiesis have been introduced into the mouse strain backgrounds, and this action should result in a lymphoid system that even more closely approximates the human lymphoid system.

Humanised mice have also been used to study (1.) haematopoietic development, (2.) a variety of microorganisms, including EBV [42, 46], herpes simplex virus [47], Dengue fever [48, 49], influenza [50] and *Salmonella typhi* [51, 52], (3.) sepsis [53] and iv) virus-induced tumours [54, 55].

Irrespective of the strain, immunodeficient mice are prone to opportunistic infections and must be kept in optimized hygienic animal care facilities. Whether the humanisation protects mice from infections is not known.

Humanised mice for studying HIV infections

The hu-PBL SCID and foetal liv/thy SCID hu mouse models have been valuable for the study of HIV infection, including immune responses (e.g., the effect of vaccination with vaccinia gp160 and recombinant gp160 [56]), *in vivo* drug testing [57–59], anti-HIV effects of CD8+ cytotoxic T-cells [60] and neutralising antibodies [61], virulence of HIV isolates [62], and the significance of distinct HIV accessory proteins on virulence [63, 64], and viral latency [65]. However, these models have several limitations. Most importantly, they lack multilineage haematopoiesis and the capacity to generate an effective human immune response (table 1).

The "new generation" of humanised mice has a number of positive aspects, such as multilineage haematopoiesis, no or very rarely graft-vs-host disease, a longer lifespan of the mice, and the generation of some immune responses (table 1). In the next sections, we will focus exclusively on these new humanised mouse models. Reviews comparing the properties of the various humanised mouse models based on the use of either Rag, NSG or NOG mouse strains have recently been published [66, 67]. In this review, we focused primarily on the overall value of humanised mice for studying HIV infection and specified only the mouse strain used when clear differences were described as related to HIV infection or pathogenesis.

Humanised mice support high-level viraemia

The new humanised mice support high levels of HIV infection with either CCR5- or CXCR4-tropic strains [36, 68–71]. Plasma HIV RNA copy numbers of 10^4–10^5/ml in those mice are similar to the levels found in HIV-infected humans (note, that HIV replication can be easily monitored by repetitive sampling of peripheral blood). HIV-infected cells were detected in the spleen, lymph nodes, thymus and lungs, indicating dissemination of the virus. Unlike hu-PBL SCID mice, humanised mice sustain high-level viral replication for more than a year [35]. Depending on the virulence of the HIV strain used, the mice show distinct CD4+ T-cell depletion rates over time. Initial reports noted either very limited or no HIV-specific humoral immune responses [68, 70]. Importantly, expression of the HIV co-receptors

CXCR4 and CCR5 on engrafted and differentiated human immune cells was similar to that seen in humans [68, 70, 71]. Co-receptor expression in human CD4+ T cells is the major determinant of HIV tropism *in vivo* [72]. Indeed, as seen in HIV-infected human, disseminated infection in humanised mice with CCR5-tropic stains leads preferentially to infection and depletion of CD4+ memory T lymphocytes [73]. CCR5 is expressed mainly on memory T lymphocytes and is absent from naive T cells.

Humanised mice for studying sexual transmission and its prevention

A prerequisite for studying HIV sexual transmission in humanised mice is the engraftment of the female reproductive tract and/or the gastro-intestinal tract with virus susceptible human cells. Both humanised Rag1-/-γ_c-/- mice and Rag2-/-γ_c -/- mice, as well as BLT mice, are well engrafted with human cells in the vagina [74–77], and vaginal HIV transmission is efficient in all these three new mouse models. Like the human gut, the mouse small intestines include abundant Peyer's patches and the large intestines are populated with lymphoid follicular aggregates with human T and B lymphocytes, macrophages and DC [43, 74]. Here, memory T cells with prominent expression of CCR5 are permissive to CCR5-tropic strains. BLT mice also show human CD4CD8αα cells, a T-cell subset present only in the gut-associated lymphoid tissue [77]. These mice respond with disseminated HIV infection subsequent to either rectal or vaginal infection with cell-free HIV [43, 74, 75, 77].

However, efficient engraftment of the gastrointestinal tract of Rag2-/-γ_c-/- mice with human cells appears to depend on the protocol used: mice transplanted with CD34+ cells derived from human foetal liver and cultured overnight with IL-3, IL-6 and stem cell factor showed human cell engraftment in the gut [74]. This is not the case in mice transplanted with uncultured CD34+ cells derived from cord blood [78]. The latter also differed in their susceptibility to rectal HIV challenge [74, 78].

Humanised mice represent a very significant advancement for evaluating novel microbicides for preventing HIV infection and very nicely complement the much more expensive monkey models. Indeed, several recent studies demonstrated the utility of these models for testing oral and topical pre-exposure prophylaxis strategies with different anti-HIV drugs (e.g., Tenofovir, Maraviroc, Raltegravir) currently on the market [77, 79–82] or with compounds in development [83]. In particular, topical application of the CCR5 antagonist Maraviroc formulated as a gel prevented HIV vaginal transmission [81], and the novel CD4 aptamer-siRNA chimeras [83] showed partial protection.

Studies of anti-retroviral treatment strategies

A few reports have noted the utility of the new humanised mice for evaluating antiretroviral therapies [84–89]. We made a major effort for defining a gold standard for ART in humanised mice by first examining the pharmacokinetic of a number of anti-retroviral compounds [90]. In this work we showed efficacious anti-retroviral treatment when the anti-retroviral compounds were added to food pellets or when long-acting drugs were used [90]. We also demonstrated emergence of resistance in insufficiently treated mice, and viral rebound from previously undetectable levels after cART interruption, confirming a latent reservoir as reported recently [85, 86, 88, 91]. Thus, humanised mice represent a highly valuable model for pre-clinical proof-of-concept studies to evaluate novel anti-retroviral compounds and to study latency that closely approximates the status of HIV-infected humans treated with cART.

Several studies also evaluated novel molecules for suppressing HIV *in vivo* in these new mouse models [89, 92–98]. They involved studies investigating the potential of Tat peptide analogues for inhibiting HIV replication (89) as

Box		
Human host factors	**Definition**	**Function**
CD4	Cluster of differentiation 4	HIV binding-entry receptor, expressed in T-helper cells, macrophages/ monocytes and dendritic cells
CCR5	C-C motif-chemokine receptor 5	Transmembrane chemokine receptor, important for cytoskeletal reorganisation and cell motility, HIV co-receptor
CXCR4	CXC motif-chemokine receptor 4	Transmembrane chemokine receptor, chemotactic activity for lymphocytes, HIV co-receptor
Cyclin CDK9	Cyclin dependent kinase 9	Cell-cycle regulator, binds Tat and cyclin T1, required for RNA polymerase II transcriptional elongation of HIV
Mouse strains	**Definition**	**Description**
C.B.-17 SCID/ SCID	Severe combined immunodeficiency (SCID)	Mutation in the *Prkd* gene (lack of B and T cells)
human-PBL-SCID	PBL: peripheral blood leucocytes	Intraperitoneal injection of human PBL into SCID mouse
Rag1$^{-/-}$ $\gamma_c^{-/-}$ Rag2$^{-/-}$ $\gamma_c^{-/-}$	Deficiency in the recombinase activating gene 1 or 2 and the common γ chain of the IL-2 receptor	Lack of B, T and NK cells
NOD	Non-obese diabetic mouse	Reduction of NK cell activity
NOD/SCID	NOD and SCID mouse	Lack of B and T cells and NK cell activity
NSG (NOD/ SCID/γc$^{-/-}$)	NOD/SCID mice with entire knock-out of the common γ_c chain receptor	Lack of B, T and NK cells and block of the maturation and activity of these cells
NOG (NOD/ SCID/γc$^{-/-}$)	NOD/SCID mice with knockout of the intracytoplasmatic tail of the common γc receptor	Lack of B, T and NK cells and block of the maturation and activity of these cells
BLT	Bone marrow, liver, thymus	NOD/SCID or NOD/SCID/γc-/- mice transplanted with foetal liver, thymus and CD34+ cells

well as the effects of silencing (si)RNAs directed against viral proteins (e.g., Tat, Rev, Vif) or CCR5 that were delivered either by aptamers binding to the HIV envelope glycoprotein [92], dendrimer nanoparticles [93], single-chain antibodies binding to CD7 [95] or by immunoliposomes targeting the lymphocyte function-associated antigen-1 (LFA-1) [94]. The gp-120-binding aptamers targeted productively infected T cells specifically, and the single-chain antibodies and immunoliposomes targeted the white blood cells independently of HIV infection. In all of these *in vivo* studies, HIV replication was significantly suppressed. The humanised mice are also useful for studying the protective effect of distinct broadly neutralising antibodies delivered either by antibody-expressing cells administered as "backpacks" [96] or by adeno-associated virus-based vectors [97].

Studying gene therapeutic approaches for HIV/AIDS

Humanised mice represent a unique option to explore haematopoietic stem cell–based gene therapy strategies. Gene manipulation of human CD34+ cells to modulate host factors is also very attractive for HIV pathogenesis studies. Immunodeficient mice have long been used to assess the ability of gene-transduced human CD34+ cells to differentiate into various cellular subsets. In 1994, retroviral vector–transduced human CD34 cells were shown to differentiate into mature T-cell subsets in SCID-hu grafts [99]. Later, long-term engraftment of human CD34+ cells transduced with an HIV vector was demonstrated in NOD/SCID mice; 4–10% of the human cells were transduced [100]. NOD/SCID and the NSG mice seem to engraft similar numbers of transduced human CD34+ cells [101]. However, when using bone marrow from those mice for secondary reconstitutions in mice on the same background, human tissue from NSG mice engrafted far better than that from NOD/SCID mice, pointing to increased numbers of long-term SCID repopulating cells. These features are favourable for studying long-term transgene expression and the analysis of retroviral-insertion sites in primary and secondary transplanted NSG mice.

These findings, along with the higher levels of human tissue engraftment, suggest that the "novel generation" of humanised mice will be very useful for studying gene therapy approaches and examining distinct genes for their pathogenic effects in various settings. In addition, zinc-finger nuclease–mediated gene engineering is another very promising gene-engineering technology that has been explored in these mouse models [102].

A vast body of literature describes various approaches to control HIV by ex *vivo* gene therapy [103, 104]. Gene therapy approaches for HIV/AIDS i) target host factors critical for HIV replication or ii) HIV genes mandatory for HIV replication (e.g., Nef) [105], iii) introduce novel gene constructs (e.g., Trim5-cyclophilin fusion protein that inhibits HIV replication) [106], or iv) employ a broadly neutralising anti-HIV antibody [107]. The value of humanised mice for studying genetically altered human CD34+ cells to treat HIV is nicely illustrated by several studies, such as those targeting CCR5 with siRNA- or shRNA-mediated silencing [108, 109] or by zinc finger–mediated excision [102]. Notably, the HIV receptor complex consists of CD4 and either the HIV co-receptor, CCR5 or CXCR4 [72]. CCR5-tropic strains are transmitted and predominate until late-stage disease [110]. CXCR4-tropic strains emerge in advanced stages of HIV disease in about 50% of HIV-infected patients and seem to accelerate the immune deficiency [111]. Silencing CCR5 has been mentioned as a potential "cure" for HIV. A recent report described an HIV-infected patient who suffered from acute myeloid leukaemia and who was transplanted with human CD34+ stem cells lacking CCR5. Strikingly, the patient appears to be fully cured since HIV did not rebound subsequent to interruption of cART [112]. While it involved only a single patient, this report gives credence to genetic approaches targeting CCR5.

Introducing a lentiviral construct silencing CCR5 into human CD34+ cells resulted in a clear reduction of CCR5 on the target cells of HIV *in vivo* [108]. The engineered progenitor cells showed long-term haematopoietic repopulating capacity by secondary transplantation. Notably, the genetically engineered progeny cells behaved identically as the controls. *In vitro* the engineered cells were resistant to HIV infection with CCR5-tropic strains. Zinc finger–mediated excision of CCR5 in CD34+ cells resulted in progeny cells lacking CCR5, and the mice showed lower HIV replication and prevention of CD4+ T-cell loss *in vivo*, as compared to control mice [102]. Very importantly, HIV infection resulted in a selection of cells resistant to HIV over time. This technology demonstrated cells with long-term haematopoietic repopulation capacity. Other genetic approaches have focused on T cells and engineered HIV-resistant CD4+ T cells with CXCR4-specific zinc finger nucleases [113].

These studies clearly indicate that humanised mice are a very promising tool for exploring gene engineering approaches to treat and/or cure HIV. The major hurdles will not be the identification of targets rendering cells resistant to HIV but to achieve sufficiently high numbers of genetically engineered CD34+ cells, the migration of transduced cells to the niches of haematopoietic stem cells, preventing the insertional risk favouring neoplastic transformation or off-target effects (e.g., activation of the innate immune response). Here, too, the humanised mouse model will be a versatile tool for exploring these questions.

Humanised mice for generating an HIV-specific immune response

Inducing a robust HIV-specific immune response was reported in NSG mice reconstituted with human CD34+ cells from newborns [114] and in the BLT mouse model [115]. Both papers reported HIV-specific CD4+ and CD8+ T-cell responses with overlapping HIV peptide pools [114, 115] or ELISPOT assays [115]. The relevance of the CD8+ T cells in constraining HIV replication in humanised mice was illustrated by a significantly higher replication rate when CD8+ T cells were depleted [114]. The humanised mice also developed humoral immune responses against HIV [115]. However, antigen-specific immune responses in humanised mice seem to take longer to develop than in adult humans infected with HIV, possibly reflecting the lack of full maturity of the human immune systems of these mice after reconstitution [115]. In previous work, such sol-

id HIV-specific antibody immune responses were not reported. The analysis might have been performed too soon after HIV infection. In view of limited number of studies, we still do not know if the current generation of humanised mice is suited for studying antigen-specific immune responses and, in particular, vaccine approaches. In mice reconstituted directly with human CD34+ cells the selection of T cells is done by murine thymic stromal cells. The subsequent generation of an antigen-specific immune response, however, is based on the processing of antigens by human antigen-presenting cells in the humanised mouse model and therefore might be suboptimal.

HIV evolution over time

HIV's diversity is one of its main features. It is also a key element for immune escape and emergence of resistance to ART. HIV's diversity is due to the inaccuracy of the HIV reverse transcriptase, hyperamination of the nascent DNA strand during reverse transcription by the members of the APOBEC family, and recombination events between distinct HIV strains. Indeed, the genotypic and phenotypic changes in the viral envelope gene in humanised mice infected with a distinct HIV strain, JR-CSF, showed the mean rate of divergence of viral populations over 44 weeks similar to that in humans [116]. They noted a disproportionate number of guanosine-to-adenosine transitions in the HIV envelope, indicating that APOBEC3G is active in this model. Furthermore, a number of substitutions in the envelope gene were identified.

HIV immune activation and dysfunction

Sustained immune activation is the major trigger of HIV-associated immunodeficiency. Various mechanisms, such as disruption of the gastrointestinal tract barrier during acute HIV infection [117] or various HIV accessory gene products, may contribute to the HIV-associated immune activation [18]. Immune activation is also observed on the CD4+ and CD8+ T cells in HIV-infected humanised mice [115, 118]. We used the humanised mice to study the role of macrophages in immune activation [118]. We found that HIV infection results in a disturbed phagocytosis by macrophages. Notably, macrophages are essential for clearing bacterial products. We concluded that disruptions of the gastrointestinal tract barrier, together with the macrophage dysfunction, are a main element of higher blood levels of bacterial products and thus in HIV-associated immune activation.

Immune activation affects also the PDL1-PD1 axis (PDL = programmed death ligand). The inhibitory receptor PD-1, which indicates exhaustion of T cells, was increased on the T cells in HIV-infected humanised mice, reminiscent of the findings in humans [115]. Ongoing studies are examining the benefits of blocking the PD-1 pathway.

The presence of various immune cells, such as plasmacytoid dendritic cells (pDC) and T-regulatory cells, in humanised mice presents a unique opportunity to assess their effects on HIV infection and vice-versa (i.e., HIV's effect on them). For example, rapid infection and activation of pDCs were seen in HIV-challenged humanised mice [119]. Their activation correlated with activation of CD4+ T cells and their apoptosis. While CD4+ T cells were depleted, pDCs were maintained but functionally impaired. The presence of T-regulatory cells in these mice may help to dissect their role in HIV infection. These cells are preferentially targeted by HIV during acute HIV infection in these mice [120].

CNS invasion by HIV

AIDS-related dementia occurs in about 30% of HIV-infected patients with advanced immunodeficiency [121]. AIDS dementia is characterised by the immigration of macrophages, formation of microglial nodules, and generation of multi-nucleated giant cells, most likely due to viral induced fusion between microglial cells and/or macrophages. HIV-infected humanised mice show pathologic anomalies in the brain reminiscent of those in HIV-infected patients with AIDS dementia. In particular, activated human blood-borne macrophages migrate into the brain. Human cells enter into the brains more quickly in HIV-infected mice than in control mice. Productively infected macrophages and cells of lymphocyte morphology are found in the meninges and perivascular spaces [122]. Strikingly, CD8+ T-cell depletion aggravated the pathological findings, suggesting that CD8+ T cells could subdue HIV infection to some extent. Using advanced neuroimaging and post-mortem examination, HIV-infected mice show a loss of neuronal integrity [123]. These data are encouraging: humanised mice represent a valuable tool for examining mechanistic and therapeutic aspects of HIV-associated dementia. However, as reiterated by the study authors, additional studies are needed for a more detailed characterisation and validation of the neuronal damage associated with HIV infection in this mouse model [124].

Future generations of humanised mice

Despite the advances made in humanised murine models, the reconstituted lymphoid system still lacks a well-elaborated lymphoid architecture. This is partially explained by the lack of human cytokines critical for haematopoiesis and/or by insufficient interactions between cells of the murine stroma and human haematopoietic cells. The less than optimal lymphoid architecture and the education/selection of T-cells on a murine thymic scaffold result in a rather modest adaptive immune response. As outlined above, transplantation of HLA-matched cord blood into a mouse strain transgenic for human HLA gives more robust specific T-cell response. To improve the humanisation of mice, additional human transgenes critical for haematopoiesis are introduced into mice.

The knock-in of human thrombopoietin (TPO) into Rag2-/-γ_c-/-, which is essential for the expansion and maintenance of HSC [125], resulted in a higher level of engraftment and an increase in the breadth of multilineage haematopoiesis with higher number of myeloid cells than in control mice expressing the murine TPO [126].

Other knock-in (KI) genes examined for improving the humanisation were human IL-3 in concert with human granulocyte macrophage cytokine stimulating factor (GM-CSF) [127] and colony stimulating factor-1 (CSF-1) [128]. In IL-3/GM-CSF KI mice, transplantation of human pregenitor cells resulted in a more pronounced inflammatory

reaction in response to intraperitoneal administration of lipopolysaccharide than in controls. In addition, IL-3/GM-CSF KI mice had improved human myeloid immune reconstitution in the lung as exemplified by the presence of alveolar macrophages; the human alveolar macrophages mounted an innate immune response when challenged with influenza virus but could not control it. This model might be especially good for studying pulmonary diseases [127]. The knock-in of CFS-1 enhanced the differentiation of myeloid cells into monocytes and macrophages [128].

As outlined above, differences in the extent the various mouse backgrounds support human cell engraftment exist. Positional cloning identified alleles of the inhibitory receptor signal regulatory protein alpha (SIRPα) as a reason for the difference of engraftment levels between mouse strains [129]. In NOD mice, which have higher engraftment levels than other mice, SIRPα on murine macrophages showed enhanced binding to the human CD47 ligand. This enhanced binding inhibits phagocytosis of the xenograft and secretion of TNF-α by macrophages. Transgenic expression of SIRPα in Rag2-/-γc-/- increased the engraftment level of HSC to a level similar to NSG mice and improved the functionality of the immune system [130].

These next-generation mice will most likely become important assets for the research community. Furthermore, the step-wise progress made in humanisation methods will continue and will create an array of humanised mouse models appropriately suited to address specific research questions.

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Figures (large format)

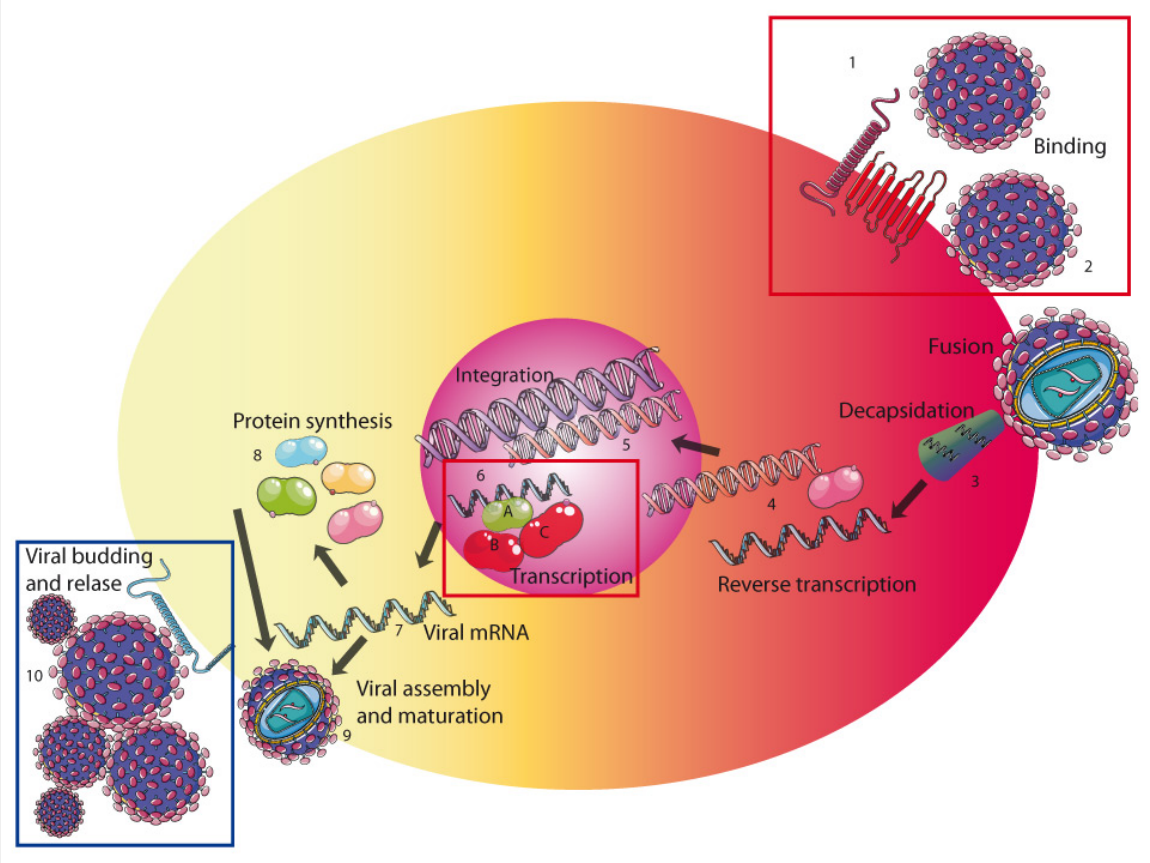


Figure 1

HIV-1 needs critical host factors for efficient replication. HIV binds to the HIV receptor complex of the human CD4 cell-surface molecule and a co-receptor, either CCR5 or CXCR4, via the HIV envelope glycoprotein 120 (HIV env gp120). After conformational changes in the HIV env gp41, viral host cell membrane fusion occurs (2). The next steps are the decapsidation (3) and release of the HIV RNA from the virus particle. Reverse transcription generates a viral complementary DNA (cDNA) based on the viral RNA template and using HIV's own reverse transcriptase (4). Once the cDNA is generated, the preintegration complex (PIC) is assembled, nuclear trafficking and integration of the viral cDNA into the host genomic DNA follow (5). Efficient transcription and elongation require formation of P-TEFb (positive transcription elongation factor b) consisting of Tat (6A), human cyclin-dependent kinase 9 (CDK-9) (6B) and cyclin T1 (6C), which binds to the nascent HIV transcripts. Fully or partially spliced HIV mRNA (7) is used to translate viral proteins. Unspliced HIV RNA is packaged into newly generated virions. Assembly of HIV proteins and RNA and budding take place at the cellular membrane (9). HIV release is inhibited by murine tetherin at the cellular membrane because murine tetherin is insensitive to the viral protein Vpu, which inhibits human tetherin by directing its proteasomal degradation (blue frame). Human host factors critical for HIV replication are CD4, CCR5, CXCR4 and cyclin T1 (red frame). Additional human specific factors probably exist.

2. Rationale and Aims

2.1 Specific Aims

1. To identify novel IFN-α dependent cellular host anti-HIV restriction factor(s) and characterize its/their molecular mechanism(s) of action.

a) To determine the permissiveness to HIV-1 infection of CD4+ T cell lines when treated with IFN-α.

b) To identify a potential IFN-α-stimulated restriction factor reducing HIV-1 permissiveness in at least one CD4+ T cell line.

c) To determine the step of the HIV viral cycle affected by the cellular factor.

2. To determine the role of the Vif-APOBEC3 axis for HIV-1 evolution *in vivo.*

a) To assess fitness, diversity and pathogenicity of HIV-1 encoding partially active Vif variants in a humanized mouse model.

b) To determine the virological and clinical relevance of APOBEC3-driven mutagenesis *in vivo*.

2.2 Rationale of this thesis

In view of the ongoing HIV-1 pandemic, the lack of a cure or a vaccine and the limitations of the currently available treatments, there is a clear need for new treatment strategies for combating HIV-1 replication. This in turn requires a deepened understanding of the HIV-1 biology.

The identification of cellular restriction factors, part of the innate immune system and critical to inhibit HIV-1 replication, may pave the way for novel treatment approaches. However, HIV-1 has acquired countermeasures, in particular by its HIV-1 accessory genes. Thus, it is important to discover new restriction factors which are able to counteract HIV-1 despite the

presence of its accessory proteins. We hypothesize that these as yet unidentified cellular factors are IFN-α-inducible. Subsequent studies may use the data from my work to generate novel therapeutic intervention strategies.

On the other hand, we hypothesize that biological properties of the Vif-APOBEC3 axis, in particular the spectrum of APOBEC3 proteins, which hypermutate the HIV-1 genome, which are neutralized by Vif, determine whether or not HIV subtypes spread rapidly in a given human population. The APOBEC3-Vif axis may be critical for therapeutic failure: HIV-1 drug resistance limits treatment options and is of great public health concern. In countries with wide access to ART, viral variants with reduced susceptibility to at least one antiretroviral drug are found in as many as 20% of newly infected patients. Thus, a narrow spectrum of APOBEC3 neutralization ability by Vif could be a risk factor for failing HAART. We consider the humanized mouse to be a very useful tool to study the role of the Vif-APOBEC3 axis for HIV-1 evolution *in vivo*.

3. Results

3.1 Analysis of the IFN-α signature by Next-Generation Sequencing in MT4 and SupT1 cells allows the identification of candidate genes for restriction factors against HIV-1

Authorship contributions

I performed all the experiments; I did all the tables, figures 1, 2, 3, 4 and 7 and supplementary figures and tables. With Annette Audigé and Roberto Speck, I designed the experiments and wrote the manuscript. Hubert Rehrauer performed the NGS data analysis and created the figures 5 and 6. This work is still a manuscript in preparation.

Introduction

Interferon-alpha (IFN-α) after binding to its cognate receptor triggers the Jak/STAT pathway, which results in a myriad of upregulated IFN-stimulated genes (ISGs)[1]. Some of these ISGs are key players in interfering with particular virus, such as MX1 with influenza[2] and other RNA viruses[3,4], but for the majority of them their biological relevance is unknown.

HIV-1 results in a prominent IFN-α response: peak viremia during acute HIV-1 infection goes along with a ~8-fold increase of IFN-α secretion[5] and subsequent upregulation of hundreds of ISGs[6]. Strikingly, the ISG signature in CD4+ T-cells from patients with acute HIV-1 infection does not differ from those with chronic HIV-1 infection[6], indicating that the ISG signature gets imprinted in the acute setting and persist thereafter, or it will be maintained by continuous IFN-α production. A very similar ISG signature also occurs in *ex vivo* HIV-1 infected primary lymphoid cultures[7,8]. Among the most common ISGs which are up-regulated in HIV-1 infection are the OAS family members (OAS1, OAS2, OAS3, OASL), the IFIT genes (IFIT1, IFIT2, IFIT3), RSAD2, the IFITM family members (IFITM1, IFITM2, IFITM3), MX1 and MX2[7,8]. The ISGs are upregulated to a different degree: for example in *ex vivo* infected lymphoid tissue, IFIT1 was upregulated 50fold, RSAD2 30fold, or APOBEC3G/F as little as 1.6 fold[7]. Notably, IFN-α released subsequent to HIV-1 challenge interferes with HIV-1 *in vivo*[9,10], *ex vivo*[11] and *in vitro*[12-14].

A limited number of the ISGs results in reduced or even abortive HIV-1 replication, especially when challenged with HIV-1 strains deficient in distinct HIV-1 accessory genes – these ISGs are called restriction factors (RFs) (reviewed in[15]). Best known examples are the

RFs APOBEC3G/F, SAMHD1 and Bst-2/Tetherin – each of those RFs act by a different mechanism (reviewed in[15]).

All the RFs identified so far are IFN-inducible. In order to develop novel treatment strategies the search for additional RFs goes on, which blocks HIV-1 despite the presence of accessory proteins and which may be exploited as anti-HIV moiety.

We hypothesized that there are additional yet unidentified IFN-α-dependent RFs and that the likelihood of ISGs to be RFs is much higher when they are present in more than one cell line where IFN-α inhibits HIV-1. Here we defined ISGs which were upregulated in two CD4+ T-cells lines where HIV-1 infection was suppressed by exogenous IFN-α treatment for defining key pathways implicated in HIV-1 suppression. We also wondered whether we are able to narrow down potential RFs/pathways by comparing the IFN-triggered signature with the HIV-triggered one assuming that the ones triggered by HIV-1 have no role as RFs as HIV-1 replicates irrespective of their presence. This work should serve as a rational basis to identify the best possible candidate genes acting as RFs in HIV-1 for their further exploration.

Materials and methods

Cell lines

MT4R5, SupT1and CEM-SS R5 cells were cultured in RPMI 1640 medium and 293 T-cells in DMEM (Sigma-Aldrich). Media were supplemented with 10% fetal bovine serum (FBS) (LONZA), 1% L-Glutamine (Life Technologies) and 1% penicillin/streptomycin (Life Technologies). SupT1 cells were transduced with the retroviral pE vector encoding CCR5 to render them permissive to CCR5 (R5)-tropic strains[16]; transduced (SupT1R5) cells were selected using a medium containing 1 μg/mL of G418 (Sigma-Aldrich).

Plasmids and viruses

Replication competent stocks of NL4-3 and YU-2 were produced by polyethylenimine (Poylsciences, Inc.) based transfection of 293T-cells with the corresponding plasmids, their subsequent precipitation with PEG-it (System Biosciences) according to the manufacturer's instructions, and their further storage at -80°C until use. The HIV-1 luciferase reporter virus pseudotyped with the envelope (env) of the ADA HIV-1 strain (ADA Env) was generated by

co-transfecting an HIV-1 proviral construct encoding a luciferase reporter gene (pNL4-3.Luc.R−E−) with an ADA Env expression vector [17] in 293T-cells.

For the generation of viral stocks free of contaminating plasmid DNA, viral stocks were produced by amplifying the NL4-3 virus in 3-way-stimulated peripheral blood mononuclear cells for 5 days[18], filtered (0.22 µm), and stored at -80°C. Virus-like particles (VLPs) consisting of the HIV-1 capsid containing Vpx protein and VSV-G envelope were produced as described in[19].

shRNAs

Custom shRNAs in the pLKO.1 vector were purchased to silence MX2 (Thermo Scientific). The non-target shRNA vector pLKO.1 was used as a negative control (Sigma-Aldrich). VLP encoding shRNAs were produced by co-transfecting the shRNA-coding plasmids in 293T with the plasmid psPAX2 encoding gag/pol and pCAG-VSVG encoding the VSV-G envelope as previously described[20]. Two days post-transfection, the supernatant was collected and viral particles were precipitated with PEG-it (see above). MT4R5 and SupT1R5 cells were transduced with the shRNAs against MX2 or non-targeted shRNA control and selected with puromycin (Sigma-Aldrich 0.3 µg/mL) for two weeks.

IFN-α treatment

For the analysis of mRNA expression of distinct ISGs, cells were treated with 1000 U/mL of interferon-alpha A/D (IFN-α) (PBL Interferon source) for 24 hours. In infection experiments, cells were pre-treated with different concentrations (0, 100 or 1000 U/mL) of IFN-α for 24 hours. IFN-α concentration was maintained in the cell culture medium. For next-generation sequencing, MT4R5 and SupT1R5 cells were pre-treated with 100 U/mL of IFN-α for 6 hours.

HIV-1 infection

YU-2 or NL4-3 was added overnight (5.7 ng of p24) to $2x10^5$ cells per well of a 96-well plate. p24 antigen concentrations were quantified using an "in-house" ELISA assay (described in[21]). 3TC (50 µM), EFV (1 µM) and T-20 (10 µg/mL) were added ~12 hours prior to infection and maintained throughout the experiment.

RNA/DNA extractions and qPCRs

RNA extractions were performed using the RNeasy Mini Kit and DNA extractions with the DNeasy Blood & Tissue Kit (both from QIAGEN). QPCRs were performed using the commercially available primers and probes for APOBEC3G, Bst-2, IFIT-1, RSAD2 and GAPDH (Hs00222415_m1, Hs00171632_m1, Hs01675197_m1, Hs00369813_m1, and Hs99999905_m1, respectively; Applied Biosystems). QPCR conditions were as follows: 95°C for 5 min, 95°C 15 sec and 60°C for 1 min (50 cycles).

For intermediate reverse transcripts, we used primers/probes for Gag regions and qPCR conditions as described previously[22]. The mean normalized gene expression was determined with the software qBase Plus 2.0 (Biogazelle).

Alu PCR

Alu PCR was performed in two steps, using a modified protocol previously described[23]. Notably, in the second round PCR, the amplification product was split in two parts for RU5- and GAPDH quantification, using the primer/probes for RU5 as previously described[22] and the primers/probes from Applied Biosystems for GAPDH detection to normalize the DNA input. As a negative control, cells were treated with the integrase inhibitor Raltegravir (Isentress, MSD MERCK SHARP & DOHME AG, 30 μM).

Western blots and antibodies

Western blot analysis was performed as previously described[24]. In order to determine the level of silencing of MX2, MT4R5 and SupT1R5 cells transduced with the shRNAs against MX2 or non-targeted shRNA control were treated with IFN-α (1000 U/mL) for 24 hours, cells were then collected and western blots performed as mentioned above.

Next-generation sequencing of total RNAs

Total RNAs were extracted from MT4R5 and SupT1R5R5 cells, in triplicates, pre-treated or not with IFN-α (100 U/mL). The RNAs were sequenced at the Functional Genomics Center Zürich (FGCZ) using the Illumina HiSeq 2000 sequencer, using two lanes of a flow cell with sample barcoding. The sequencing library was prepared as single stranded using the TruSeq protocol (Illumina), which includes poly-A selection. Reads were sequenced as paired-end 100+100. Expression values were computed with RSEM[25]. As reference the human genome build GRCh37 from Ensembl was used as a reference. A list of differentially expressed genes which are potential RFs was obtained by comparing the IFN-α treated samples with the

corresponding untreated samples for each cell line. The statistical test was conducted with the DESeq2 [26] package in Bioconductor.

Gene ontology and enrichment analysis

Enrichment analysis consists of matching genes in functional ontologies by GeneGo Meta-Core version 6.19. The 162 common upregulated genes there uploaded to MetaCore for pathway and network analysis. MetaCore was used to build a network from the 162 genes by using a shortest path approach and by adding at most two nodes to link two of the genes.

Statistics

For statistical analysis, the statistical software provided by GraphPad Prism (Version 5.04) was used. The significance level of $p < 0.05$ was considered as relevant. In the figures, p-values are presented for comparisons between treatment groups and controls and are denoted by asterisks.

Results

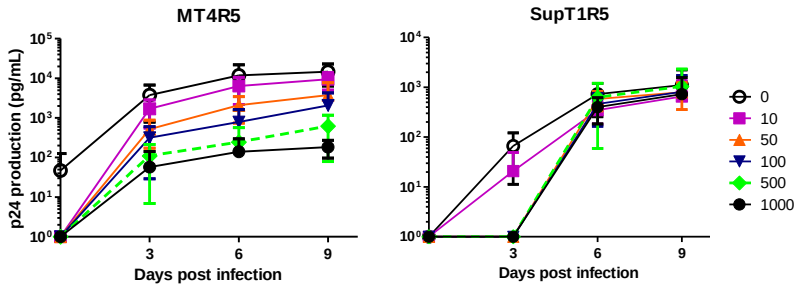
MT4R5 and SupT1R5 but not CEM-SS cells are highly responsive to IFN-α

We explored IFN-α responsiveness in MT4R5, SupT1R5 and CEM-SS cell lines by quantifying the prototype ISGs IFIT-1 and RSAD2 (Viperin) as well as APOBEC3G and Bst-2. We observed a vigorous up-regulation already 6 hours after adding IFN-α in MT4R5 and SupT1R5 cell lines which persisted over the next 24 hours (Suppl. Fig. 1 and 2). In contrast, CEM-SS reacted only poorly. Thus, MT4R5 and SupT1R5 cell lines had the prerequisites for studying ISGs.

IFN-α inhibits HIV-1 replication in MT4R5 and SupT1R5 cells

We observed a clear dose-dependent anti-HIV effect of IFN-α with nearly complete inhibition at 100 and 1000 U/ml in MT4R5 cells while a less prominent one in SupT1R5 cells (Fig. 1a and b). At 3 days of HIV-1 infection, the inhibitory concentration (IC) 50 was 10 U/mL for both cell lines.

A



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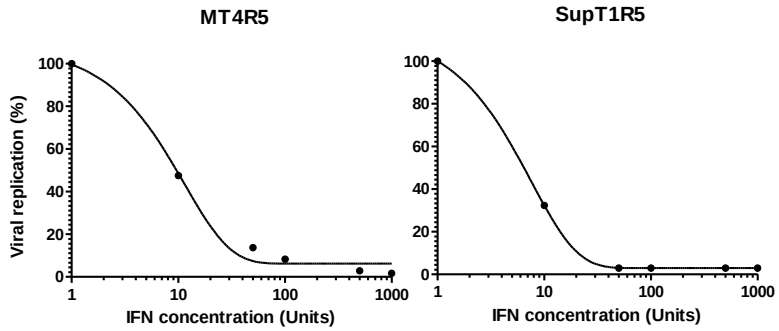

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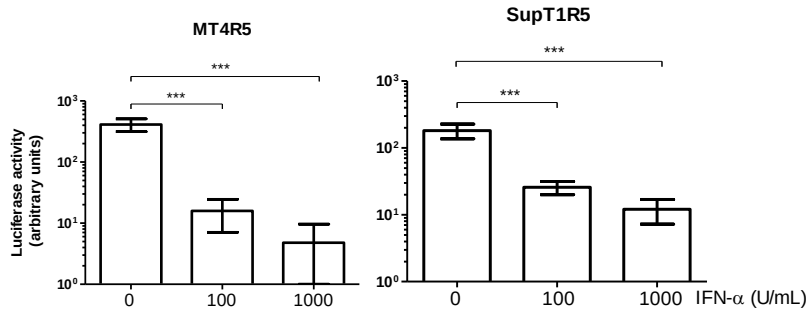

Figure 1: HIV-1 replication is strongly inhibited by IFN-α in MT4R5 and SupT1R5 cells. (A) Viral replication in MT4R5 cells (left panel) and SupT1R5 cells (right panel) over time. Cells were pre-treated with different concentrations of IFN-α (ranging from 0 to 1000 U/mL) for 24 hours and infected with the CCR5-tropic HIV-1 strain YU-2. IFN-α was replenished every three days. HIV-1 replication was monitored by measuring the p24 antigen concentration (pg/mL) in the culture supernatant on days 0, 3, 6 and 9 post-infection. Each dot corresponds

to the average of three experiments performed in triplicates and the bars indicate the standard deviation (SD). (B) Dose-response curve to define the IC50 of IFN-α in HIV-infected MT4R5 cells (left panel) and SupT1R5 cells (right panel). HIV replication between days 0 and 3 from (A) was used to calculate the area under the curve as an indication of viral replication. (C) Luciferase activity in MT4R5 cells (left panel) and SupT1R5 cells (right panel) pre-treated with IFN-α after challenge with a luciferase reporter virus (n=3; average and SEM shown). Statistics were performed using a one-way analysis of variance test and a Dunnett's post test. *** represents $p<0.001$

We corroborated these results with replication incompetent viral particles encoding the firefly luciferase gene and pseudotyped by the R5-tropic ADA envelope. We observed on average a 26- and 86-fold decrease of luciferase activity in MT4R5 cells and a 7- and 15-fold decrease in SupT1R5 cells at 100 and 1000U/ml of IFN-α respectively (Fig. 1c).

IFN-α interferes mainly after reverse transcription but prior to integration

To further dissect at what step IFN-α interferes with HIV-1 replication, we measured intermediate reverse transcripts as well as integrated HIV-1 DNA. We used here the parental cell lines instead the R5 complemented ones since CCR5 was introduced into the cells using lentiviral based transduction and their remnants might confound the PCR assays used. IFN-α inhibited the CXCR4-tropic strain NL4-3 in MT4 cells similarly as it inhibited YU-2 in the MT4R5 ones which justified and validated the use of the parental cell lines for that purpose (Suppl. Fig. 3). IFN-α decreased proviral DNA in MT4 cells dose-dependently by 11-fold at 100 U/mL, and 343-fold at 1000 U/mL (Suppl. Fig. 4a). The effect was less pronounced for SupT1 cells with a 1.3-fold reduction at 100 U/mL and 2.3-fold reduction at 1000 U/mL respectively (Suppl. Fig. 4b). We also observed a significant decrease for intermediate reverse transcripts (Gag) for the IFN-treated MT4R5 cells (Suppl. Fig.. 4c) and SupT1 cells (Suppl. Fig. 4d).

The rather complete inhibition of HIV-1 particle production at higher dosage of IFN-α while the less than complete inhibition of RT products points to IFN's activity at various stages in the HIV-1 replication cycle. Since we infected the cells with wild-type HIV-1, budding will most likely not be due to Tetherin, which is counteracted by Vpu[27].

Functional characterization of the ISGs

To identify genes potentially implicated in the IFN-mediated block in MTR54 and SupT1R5 cells, we did NGS of mRNA from cells treated with IFN-α for 6 hours. We considered genes as significantly changed if $p < 0.01$. For the subsequent functional analysis we required additionally 2-fold expression change. We found 396 genes up- and 15 genes downregulated in MT4R5 and 304 genes up- and 15 genes downregulated in SupT1R5 using the Illumina NGS platform. We identified 162 genes, which were upregulated in both cell lines simultaneously and none which was downregulated according to these criteria (Fig. 2, left panel and Suppl. Table 1). In comparison to previous analysis done in IFN-α exposed MT4 cells using microarray[28], we observed a high correlation but generally larger fold-changes and higher significances for the upregulation which indicates a higher sensitivity of our NGS based expression assay.

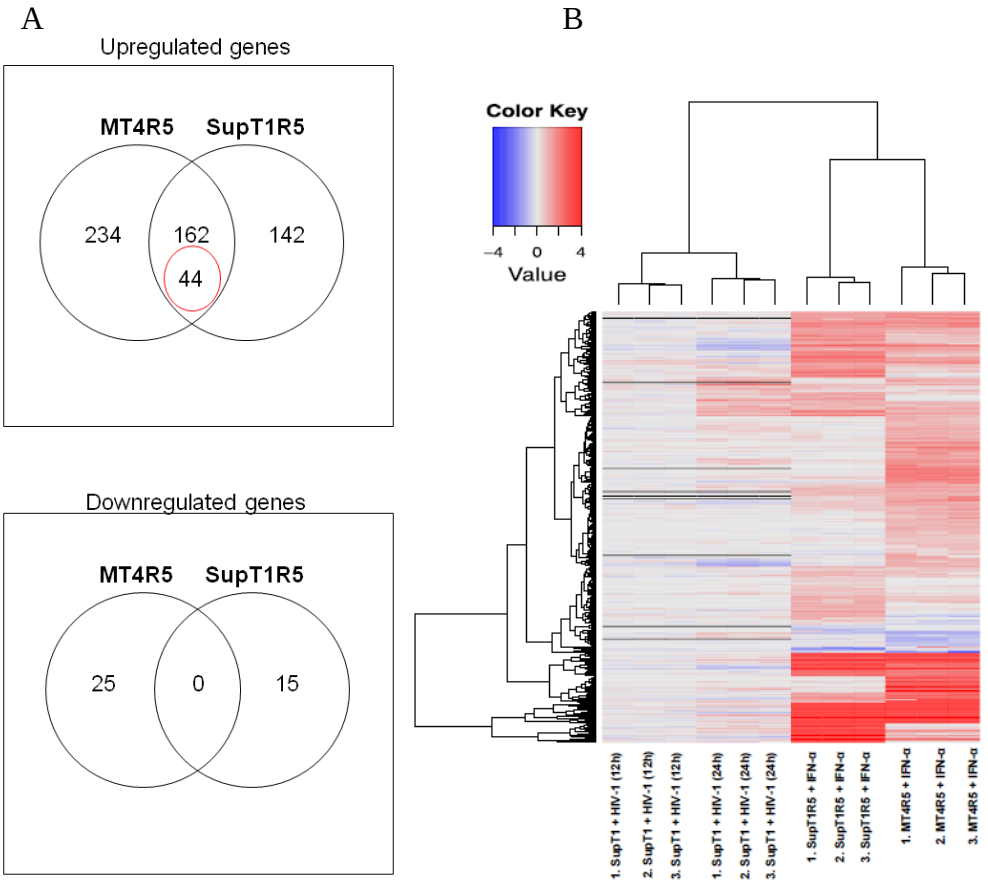

Figure 2. IFN-α treatment results in a prominent ISG signature in MT4R5 and SupT1R5 cells but only in a faint one in HIV-infected cells. (A) The number of upregulated vs downregulated genes in MT4R5 and SupT1R5 cells is depicted here. The number of genes

which are at once upregulated in our dataset as well as in the literature is indicated with a red circle (left panel). (B) Genes representing the gene signature in SupT1 cells 12 and 24 hours after HIV-1 infection and gene signature from MT4R5 and SupT1R5 cells after IFN-α treatment The similarity of gene expression profiles among experimental samples is summarized in a dendrogram above the cluster heat map, in which the pattern and the length of the branches reflect the relatedness of the samples. Upregulated genes are represented in red, genes with no fold change are represented in white and downregulated genes are represented in blue.

A previous report using HIV-infected SupT1 cells and total RNAseq analysis revealed only limited genome-wide changes[29] (Fig. 2, right panel). All of those changes were also present in our analysis which suggests that HIV-1 triggered changes are indirect by triggering the IFN-α axis.

We then investigated to what extent the genes which were upregulated in both cells lines could be categorized to certain regulatory pathways/networks in MetaCore™ (Thomson Reuters v.6.19), i.e., canonical pathway maps, GO (gene ontology) and network processes.

Canonical pathway maps showed that the genes upregulated in both cell lines were attributed to 10 different canonical pathway maps (from 260 existing ones) and were, as expected, predominantly immune response genes (Suppl. Table 2). In this pathway map analysis, we had 10 genes with unknown anti-viral effects, but with known molecular function; 5 genes with a known anti-HIV activity, and 9 genes with an anti-viral activity which have not yet been explored for their potential effects on HIV-1 (Suppl. Table 2). Some of these genes such as STAT1[30], STAT2[30] and IRF9[31] are rather signal transducers or transcriptional factors, which will not be taken into account for further analyses. Of note, only 24 out of the 162 genes upregulated in both cell lines were taken into account in the canonical pathway maps; for the other 138 genes no pathway information is available in the MetaCore databsase. Notably, genes with unknown function cannot be taken into account in any functional classification system. The limited value of the canonical pathway map analysis is also demonstrated by the lack of the known HIV-1 RF, such as APOBEC3G, Bst-2, SAMHD1, TRIM5, etc. showing up.

Subsequently, we looked at MetaCores categorization of the genes according to GO processes (Suppl. Table 3) and the Process networks and obtained a large number of genes accumulated in the different processes (Suppl. Table 4). From the 162 genes upregulated in both cell lines, 76 genes were present in the GO processes. 37 genes were present in the Process networks and similarly to the gene present in some particular process. Like the canonical pathway maps, these analyses also showed that the upregulated genes in both cell lines were predominantly immune response genes. Although the known RF mentioned above (APOBEC3G, Bst-2, SAMHD1, TRIM5, etc.) were present in GO processes, they were not present in the process networks (except for MX2). The different known RFs were not accumulated in a particular GO process, but rather in almost each single process, together with several other genes (between 27 to 70 genes present in each single GO process).

44 of the 162 IFN-α upregulated genes are consistently reported in the literature

Since the distinct functional classifications did not point to a particular process, we looked for an alternative approach to analyse our NSG data: we compared the 162 IFN-α upregulated genes with data in the literature, which report genes upregulated either by IFN-α treatment[32] or, by HIV-1 triggered release of IFN-α in *in vitro* settings[7,8] and genes upregulated from patient specimens obtained from patients with acute or chronic HIV-1 infection[6,43-46]. The literature search was performed using the keywords "IFN-α AND gene expression" or "HIV-1 AND gene expression" in the PubMed database (Suppl. Table 5). This approach was built upon our original working hypothesis that ISGs able to block HIV-1 will come up in more than one cell system.

We considered genes as relevant for our analysis which were reported to be upregulated in at least three different publications; 53 met these criteria, and 44/53 were also upregulated in our dataset – less stringent criteria, e.g., twice mentioning of genes of interest, would have faced us with even a higher number of potential candidates (more than 81) (Suppl. Table 5). We found that i) 13/44 genes are anti-viral active, but their anti-HIV activity has yet to be studied; ii) 20/44 genes with reported anti-HIV-1 activity; iii) 11/44 genes without any known antiviral activity or which are intermediate signalling but certainly not effector molecules (e.g., STAT1[33], IRF9[34]), or which increase viral replication (LY6E[35])(Fig. 3).

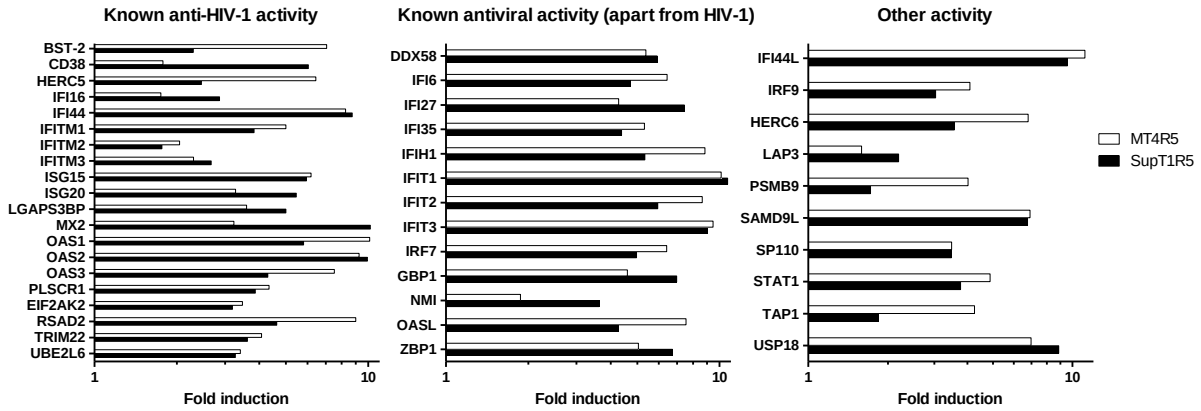

Figure 3. The 44 genes, which were at once upregulated in our dataset and in related studies in the literature belong either to genes with known anti-HIV function, known anti-viral activity and unknown activity.

SAMHD1 nor MX2 play a role in the IFN-mediated HIV-inhibition in MT4R5or SupT1R5 cells.

SAMHD1 is an IFN-type 1 and 2, MX2 a solely IFN-type 1 up-regulated RF – they act either during reverse transcription[36] or prior to integration[37,38], respectively, and are among the 162 upregulated genes we found (Suppl. Table 1) and were also coming up in the genes enriched in the biological processes (Suppl. Table 3). Thus, we explored the role of SAMHD1 and MX2 in the IFN-α mediated effects by degrading SAMHD1 by Vpx (Fig. 4) and MX2 by knocking it down in the cell lines (Suppl. Fig. 5). We observed no reversal of the IFN-mediated anti-HIV activity that negates any role of SAMHD1 and MX2 in the observed IFN-α-mediated inhibition of HIV-1 (Fig. 4).

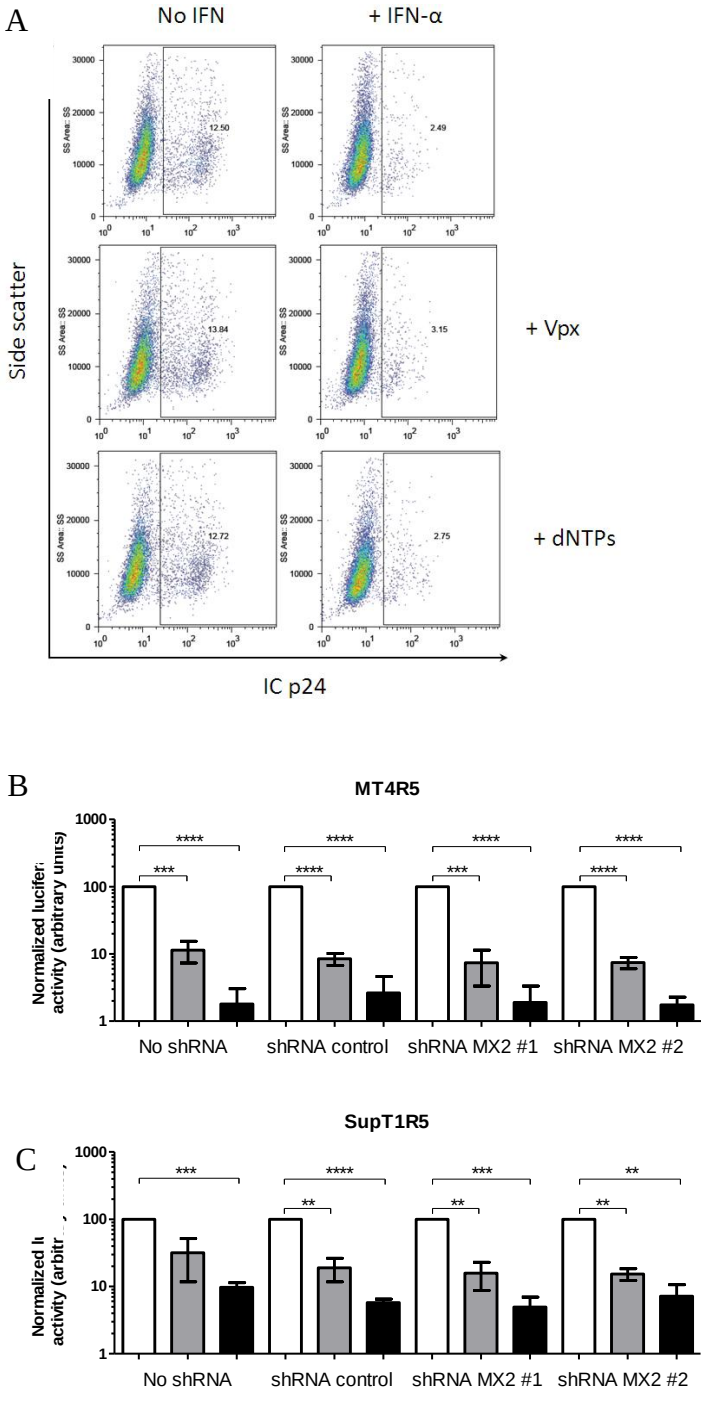

Figure 4. Neither SAMHD1 nor MX2 had any role in the IFN-mediated HIV-inhibition in MT4R5 and SupT1R5 cells. (A) MT4R5 cells were treated with 0 or 1000U/mL of IFN-α (left panel and right panel, respectively). 22 hours later, cells were treated either with VLP containing the Vpx protein (second row) or with dNTPs (third row) and 2 hours later infected with HIV-1 YU-2. 48 hours post-infection, cells were stained for intracellular (IC) p24 expression. Data shown correspond to a single experiment representative of 3 independent

experiments. (B) Luciferase activity in the different MT4R5 stably cell lines generated with MX2 shRNAs after infection with a luciferase reporter virus and treatment with different IFN-α concentrations (left, middle and right bars: 0, 100 and 1000 U/mL). (C) Luciferase activity in the different SupT1R5 stably cell lines generated with MX2 shRNAs after infection with a luciferase reporter virus and treatment with different IFN-α concentrations (0, 100 and 1000 U/mL). Each bar corresponds to the average of three experiments. Error bars correspond to SEM. Statistics were performed using a one-way analysis of variance test and a Dunnett's post test. ** represents $p<0.01$, *** represents $p<0.001$ and **** represents $p<0.0001$.

Discussion

We argued that key pathways or effector genes switched on by IFN-α and inhibiting HIV-1 are likely the same in different cell lines and that this approach would permit to identify unknown RF. Using MT4R5 and SupT1R5 cells, we found that i) IFN-α blocked HIV-1 between reverse transcription and proviral integration; ii) the functional classifications of MetaCore did not provide a definitive subset of RF candidates; iii) a comparison of the genes upregulated in our cell lines and the literature identified a subset of 44 RF candidate and iv) neither MX2 nor SAMHD1 were responsible for the inhibitory effect of IFN-α observed. Thus, we still are challenged with a large number of potential hits, which needs their individual testing in the appropriate setting.

APOBEC3G, TRIM5α, SAMHD1, BST2 and MX2 were discovered by comparing HIV-1 permissive vs. HIV-resistant cells[27,39-42]. Another approach was based on a shRNA-based screen to identify proteins necessary for HIV-1 replication[43-45]. The latter approach gave a large number of hits; the significance of each would require functional testing. A very recent work reported the use of evolutionary genomic signatures and functional analyses for the identification of RFs[46]. We approached the issue of identification of RFs from another angle - we assumed that the same ISGs would interfere with HIV-1 replication in different HIV-1 permissive cell lines treated with IFN-α and by that way may point to potential RF candidates. We have chosen the highly IFN-α responsive cell lines, MT4R5 and SupT1R5. Consistent with previous reports[47], the anti-HIV-1 activity by IFN-α was strong and sustained in MT4R5 cells but less prominent and only transient in SupT1R5. This discrepant effect might be

explained that SupT1R5 cells gets refractory or exhausted shortly after IFN-α exposure. Supporting this, we found that IRF2, which inhibits IRF1, which in turn promotes an anti-HIV state[35] and SOCS1, which attenuates the IFN-α axis[48] are significantly upregulated in SupT1R5 but not in MT4R5 cells (data not shown).

IFN-α blocked HIV-1 replication at the level of reverse transcription, but prior to integration, in the cell lines used, similar to IFN-α's anti-HIV activity in primary CD4+ T-cells[47] – the block at the same level of replication validates the use of our cell lines. For studying the IFN-α signature in these two cell lines, we argued that 100 U/ml is optimal since it was the lowest dose with the maximum antiviral effect (IC90) and 6 hours because at that time point the IFN-α signature is already deployed[49], and we wanted to assess the first wave of ISGs.

We first compared the IFN-α signature in SupT1R5 cells with the one reported in SupT1 cells infected with HIV-1. In line with previous reports[6,50,51], we found a very prominent ISG signature in SupT1 cells triggered by IFN-α while only a very faint one in HIV-1 infected cells. HIV-1 resulted in other genome-wide changes regarding apoptosis-related genes[7], Toll/IL-1 pathway and pulmonary resistance[52]. The very faint expression of ISGs in HIV-1 infected T-cells is not surprising since IFN-α production is defective in HIV-infected T-cells and macrophages[53,54]

162 genes were upregulated in both cell lines subsequent to IFN-α treatment. Of note, the well-known RFs, APOBEC3G, Tetherin/Bst-2, SAMHD1, TRIM5α and the recently identified MX2 were among those genes which supports our assumption. However, these RFs were represented to a varying extent in the various functional classifications by MetaCore, and very importantly, they were distributed all over the various biological processes, and thus did not permit focussing on a particular process.

We also had a look at the genes which were upregulated either in the MT4R5 or SupT1R5 cells. They had no known antiviral activity except for AIM2 (against Vaccinia virus[55]) in MT4R5 cells and DDX60 (interferon-inducible gene in response to viral infection[56]) and PARP12 (against Venezuelan-equine encephalitis virus[57]) in SupT1R5 cells.

In order to position our data and to narrow down the list of potential RFs candidates, we compared the 162 upregulated genes to previous analyses looking either at studies examining

the effects of exogenous IFN-α treatment[32] or examining the effects solely upon HIV-1 infection *ex vivo* (primary PBMCs[7] or primary MDM[8]) or analyses of genes upregulated from PBMCs from rhesus macaques[52] (chronic SIV/HIV-1 chimeric infection) or CD4+ T-cells from HIV-infected patients (chronic or acute HIV-1 infection)[6,58-60]. Indeed, the overlapping gene pattern of the different studies was close to the one we had in the two cell lines (Suppl. Table 5). We obtained 44 genes which were upregulated in the literature as well as in our cell lines. Of note, the least similar pattern to what we have was observed for primary MDM[8], for reasons mentioned above. This approach seems to be valid since we identified a great majority of anti-HIV active genes (including well characterised RF) (20/44) and antiviral genes (12/44) and also since several known RFs (Bst-2, MX2, PKR, RSAD2, etc.) were included in this selection of genes, except for APOBEC3G, SAMHD1 and TRIM5α. We believe focusing on those genes would make sense for searching for HIV-1 RFs. Coming back the recent report using evolutionary genomic signatures and functional assays[46], the researchers found new potential anti-HIV activity for IFI44L, APOL3 and APOL6. Interestingly, IFI44L is present in our list of 44 genes concomitantly upregulated in the literature (classified as "other activity") and APOL3 and APOL6 are present in the162 upregulated genes.

Irrespective of the approach we use to analyse our data, we will eventually be challenged with functional testing of the RF candidates. The major question remains: How to identify the particular function of a gene as related to HIV-1? Indeed, MX2 and SAMHD1 were upregulated in MT4R5 and SupT1R5 cells. Since they act prior to proviral integration[42,61], we considered them as promising RFs in our cell system; but this was not the case, pointing to another IFN-dependent mechanism(s) of HIV-1 inhibition in our cell system. Thus, testing candidate genes in the "wrong" assay system will result in false negative results.

In summary, with our approach, looking at the IFN-α signature in two HIV-permissive cell lines, we obtained 162 ISGs which were present at once and which are potential RF candidates. By including other genome wide analyses of HIV-infected tissue, we defined a subset of 44 genes which we consider the most promising candidates to look at first.

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Authorship contributions

GGH, AA and RFS designed the experiments and wrote the manuscript. GGH performed the experiments. HR performed the gene enrichment analysis.

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Supplementary Material

Supplementary Table 1: Genes upregulated by IFN-α in MT4R5 and SupT1R5R5

Gene name	Fold upregulation in		Gene name	Fold upregulation in		Gene name	Fold upregulation in		Gene name	Fold upregulation in	
	MT4R5	SupT1R5		MT4R5	SupT1R5		MT4R5	SupT1R5		MT4R5	SupT1R5
IFIT1	10.7	10.14	C19orf66	3.774	3.938	MYD88	2.32	1.665	NLRC5	1.628	5.058
MX2	10.18	3.226	STAT1	3.771	4.876	BST2	2.296	7.041	DLL1	1.587	3.231
OAS2	9.924	9.261	PHF11	3.755	2.701	RARRES3	2.289	3.56	HCG4P11	1.544	2.485
IFI44L	9.578	11.15	NMI	3.63	1.872	HAPLN3	2.226	2.574	CHST12	1.533	1.463
IFIT3	9.023	9.453	TRIM22	3.62	4.076	LAP3	2.194	1.588	NAPA	1.527	1.434
USP18	8.87	6.968	SMTNL1	3.619	2.809	VSIG10L	2.192	1.558	RBM43	1.508	1.726
IFI44	8.731	8.26	C5orf56	3.618	3.541	GBP3	2.186	4.143	RP11-	1.501	1.352
EPSTI1	7.968	9.458	HERC6	3.567	6.801	PNPT1	2.173	1.972	TREX1	1.495	1.556
XAF1	7.936	6.561	CTD-	3.527	3.58	CTD-	2.172	1.876	TRIM14	1.445	1.768
IFI27	7.433	4.28	SP110	3.483	3.484	CTD-	2.124	5.909	TRIM56	1.414	1.379
GBP1	6.97	4.603	CD68	3.423	3.325	APOL2	2.083	2.131	APOBEC	1.405	1.345
SAMD9L	6.766	6.903	AC00995	3.416	3.025	LY6E	2.075	3.765	CMTR1	1.387	1.31
ZBP1	6.729	5.052	NT5C3A	3.408	1.525	GMPR	2.031	1.881	CTD-	1.383	1.549
CD38	6.04	1.776	HSH2D	3.382	4.231	RP11-	2.006	2.527	CD274	1.382	2.007

IFIT5	5.981	8.474	RN7SL83	3.375	2.649	RBCK1	1.988	1.59	TTC21A	1.381	1.911
ISG15	5.944	6.176	UBE2L6	3.271	3.408	NUB1	1.967	1.65	SP140	1.31	1.562
IFIT2	5.942	8.637	PARP10	3.204	5.262	TRIM25	1.905	2.306	C2CD2	1.304	1.703
DDX58	5.912	5.374	EIF2AK2	3.19	3.466	SAMHD1	1.889	3.419	HES4	1.301	1.219
OAS1	5.801	10.11	HMGB1P3	3.168	3.208	MOV10	1.882	2.395	HLA-F	1.287	2.181
RP11-288L9.4	5.57	6.539	SSTR3	3.143	3.921	SLFN5	1.879	1.639	LGALS9	1.285	5.684
ISG20	5.458	3.274	NT5C3A	3.083	1.44	TAP1	1.842	4.255	CD164	1.264	1.126
IFIH1	5.33	8.842	AC116366.6	3.073	3.149	RP11-799D4.4	1.829	1.332	CTD-2047H16.4	1.244	1.756
LGALS3BP	4.995	3.59	IRF9	3.03	4.091	UNC93B1	1.824	1.884	ZNFX1	1.208	1.634
IRF7	4.971	6.405	STAT2	3.009	3.694	TAP2	1.823	2.329	RASGRP3	1.19	4.582
TRANK1	4.801	2.99	STAP1	2.986	2.065	SLC8A2	1.821	1.745	RNF213	1.187	4.091
IFI6	4.711	6.427	PRICKLE1	2.965	1.702	WDR86	1.807	2.235	PFKFB4	1.187	1.62
SIDT1	4.676	1.061	APOL6	2.894	6.277	PTGIR	1.799	1.458	N4BP1	1.186	1.918
RSAD2	4.628	8.988	IFI16	2.857	1.748	CHMP5	1.792	1.727	TRIM34	1.179	3.426
IFI35	4.384	5.31	APOL3	2.796	2.616	TRIM5	1.782	2.076	CEACAM1	1.173	1.249
RP11-662G23.1	4.37	3.642	IFITM3	2.669	2.302	TDRD7	1.774	2.679	PI4K2B	1.164	1.358
OAS3	4.29	7.509	REC8	2.666	3.886	TNK2	1.764	1.708	SPATS2L	1.163	2.102
OASL	4.266	7.542	TNK2-AS1	2.657	2.614	IFITM2	1.762	2.047	PSME2	1.161	1.681

RP11-1094M14.8	4.225	1.724	RP11-686D22.8	2.589	4.389	AP000867.1	1.74	1.094	GSDMD	1.154	1.455
OR52K3P	4.13	6.198	C3AR1	2.567	2.298	OTOF	1.737	2.15	RP11-23P13.7	1.135	2.171
HELZ2	4.018	5.627	SP100	2.531	3.061	PSMB9	1.719	4.023	ERAP2	1.123	1.077
SLFN12	4.008	2.963	TRIM69	2.517	2.596	SP140L	1.715	1.654	EHD4	1.116	2.317
DHX58	3.913	5.11	TRIM21	2.49	3.879	ADAR	1.702	1.888	AC004840.9	1.098	1.46
PLSCR1	3.868	4.335	HERC5	2.459	6.427	IL15RA	1.701	2.008	SPTBN5	1.095	2.613
SAMD9	3.828	3.764	CTD-2521M24.9	2.428	4.42	PML	1.666	2.527	TAPBP	1.094	1.306
IFITM1	3.821	4.996	SLC2A12	2.409	1.964	PPM1K	1.649	1.695	GBP2	1.082	1.762
									BTN3A3	1.072	1.291
									TTC38	1.065	1.159

Supplementary Table 2. MetaCore canonical pathway maps analysis that depicts pathway of the genes characterizing the response to IFN-α in MT4R5 and SupT1R5R5.

Maps	p-value	Genes from active data
Immune response IFN-α/β signaling pathway	5.529E-17	IFI6, STAT2, IRF9, ISG54, STAT1, PML, USP18, STAT1/STAT2, ISG15
Immune response Antiviral actions of Interferons	7.558E-15	PKR, OAS1, STAT2, OAS3, IRF9, ADAR1, OAS2, STAT1
Immune response Innate immune response to RNA viral infection	6.168E-07	IRF7, MDA-5, RIG-I, TLR3, LGP2
Immune response Role of PKR in stress-induced antiviral cell response	2.298E-05	IRF7, MyD88, PKR, TLR3, STAT1
Development Angiotensin signaling via STATs	3.909E-05	STAT2, IRF9, STAT1
Immune response IFN-γ signaling pathway	3.112E-04	PKR, IRF9, STAT1
Immune response IL-15 signaling via JAK-STAT cascade	3.476E-04	STAT2, IL-15RA
Immune response Antigen presentation by MHC class I	6.293E-04	Tapasin, PSME2, TAP1 (PSF1)
Development Prolactin receptor signaling	5.210E-03	OAS1, NMI, STAT1
Development Thrombopoetin signaling via JAK-STAT pathway	7.783E-03	TAP1 (PSF1), STAT1

Genes with anti-HIV activity
Genes with antiviral activity (excluding HIV-1)
Genes with no direct antiviral activity or other functions

Note: p-value represents the probability that the genes are included in each pathway by chance

Supplementary Table 3. MetaCore gene ontology (GO) cellular processes analysis that depicts functional ontologies of the genes characterizing the response to IFN-α in MT4R5 and SupT1R5R5.

GO Processes	p-value	Genes from active data
Defense response to virus	6.7607E-63	NLRC5, BST2, IRF7, PKR, MDA-5, IFI17, RIG-I, EFP, TRIM56, TLR3, IFI16, OAS1, SAMHD1, STAT2, APOBEC3G, Ceb1, OAS3, GBP1, IFITM3, RI58, IRF9, OASL, MxB, UNC93B, IFITM2, ISG54, RSAD2, IFI56, ADAR1, ZBP1, OAS2, ISG20, IFIT1, STAT1, RIG-G, PML, GBP3, Staf-50, PL scramblase 1, STAT1/STAT2, DDX60, ISG15, LGP2, TRIM5
Response to virus	3.0841E-53	NLRC5, BST2, IRF7, MyD88, PKR, MDA-5, IFI17, RIG-I, EFP, TRIM56, TLR3, IFI16, OAS1, SAMHD1, STAT2, APOBEC3G, Ceb1, OAS3, GBP1, IFITM3, RI58, IRF9, OASL, MxB, UNC93B, IFITM2, ISG54, RSAD2, IFI56, ADAR1, ZBP1, OAS2, ISG20, CXCR4, IFIT1, H28, STAT1, RIG-G, PML, GBP3, Staf-50, PL scramblase 1, IFI44, STAT1/STAT2, DDX60, ISG15, LGP2, TRIM5
Defense response to other organism	8.1402E-47	NLRC5, BST2, IRF7, MyD88, PKR, MDA-5, IFI17, RIG-I, EFP, TRIM56, TLR3, IFI16, OAS1, SAMHD1, STAT2, APOBEC3G, Ceb1, OAS3, GBP1, IFITM3, RI58, IRF9, OASL, MxB, UNC93B, IFITM2, ISG54, RSAD2, IFI56, ADAR1, ZBP1, OAS2, ISG20, IFIT1, H28, STAT1, RIG-G, PML, GBP3, Staf-50, GBP4, PL scramblase 1, GBP2, STAT1/STAT2, DDX60, ISG15, LGP2, TRIM5
Response to type I interferon	8.2312E-46	IRF7, SP100, IFI17, TRIM56, IFP 35, OAS1, IFI6, STAT2, OAS3, HLA-F, IFITM3, IRF9, OASL, MxB, IFITM2, ISG54, IFI56, ADAR1, OAS2, ISG20, XAF1, IFI27, IFIT1, STAT1, RIG-G, USP18, GBP2, STAT1/STAT2, ISG15
Innate immune response	1.7585E-44	UBCH8, NLRC5, TREX1, BST2, IRF7, MyD88, SP100, PKR, MDA-5, IFI17, RIG-I, NUB1, EFP, MOV-10, TRIM56, IFP 35, TLR3, IFI16, OAS1, IFI6, SAMHD1, TRIM14, SP110, HERC6, STAT2, APOBEC3G, Ceb1, OAS3, GBP1, HLA-F, IFITM3, RI58, IRF9, OASL, MxB, UNC93B, IFITM2, ISG54, RSAD2, IFI56, ADAR1, ZBP1, OAS2, ISG20, XAF1, IFI27, IFIT1, STAT1, RIG-G, PML, USP18, GBP3, Ro52, GBP4, GBP2, STAT1/STAT2, DDX60, ISG15, LGP2, TRIM5
Cellular response to type I interferon	4.7734E-44	IRF7, SP100, IFI17, IFP 35, OAS1, IFI6, STAT2, OAS3, HLA-F, IFITM3, IRF9, OASL, MxB, IFITM2, ISG54, IFI56, ADAR1, OAS2, ISG20, XAF1, IFI27, IFIT1, STAT1, RIG-G, USP18, GBP2, STAT1/STAT2, ISG15
Immune effector process	1.0615E-43	NLRC5, BST2, IRF7, MyD88, PKR, MDA-5, IFI17, RIG-I, EFP, BTN3A3, TRIM56, TLR3, IFI16, OAS1, SAMHD1, STAT2, APOBEC3G, Ceb1, OAS3, GBP1, IFITM3, RI58, IRF9, OASL, MxB, UNC93B, IFITM2, ISG54, RSAD2, IFI56, ADAR1, ZBP1, OAS2, ISG20, IFIT1, H28, STAT1, RIG-G, PML, GBP3, Staf-50, PL scramblase 1,

		STAT1/STAT2, DDX60, ISG15, LGP2, TRIM5
Immune response	2.1907E-43	UBCH8, NLRC5, Tapasin, TREX1, BST2, IRF7, MyD88, SP100, PKR, MDA-5, IFI17, RIG-I, NUB1, EFP, MOV-10, BTN3A3, TRIM56, IFP 35, TLR3, IFI16, OAS1, IFI6, SAMHD1, TRIM14, SP110, CD164, HERC6, STAT2, APOBEC3G, Ceb1, OAS3, GBP1, HLA-F, IFITM3, RI58, IRF9, OASL, MxB, UNC93B, CCR1, IFITM2, ISG54, RSAD2, IFI56, ADAR1, ZBP1, OAS2, ISG20, XAF1, C3aR, IFI27, IFIT1, H28, STAT1, RIG-G, PML, USP18, GBP3, PD-L1, Ro52, Staf-50, GBP4, PL scramblase 1, GBP2, STAT1/STAT2, DDX60, ISG15, LGP2, TRIM5
Defense response	4.4943E-43	UBCH8, NLRC5, 90K, Tapasin, TREX1, BST2, IRF7, MyD88, SP100, PKR, MDA-5, IFI17, RIG-I, NUB1, EFP, TAP1 (PSF1), MOV-10, TRIM56, IFP 35, TLR3, IFI16, OAS1, IFI6, SAMHD1, TRIM14, SP110, APOL3, HERC6, STAT2, APOBEC3G, Ceb1, OAS3, GBP1, HLA-F, IFITM3, RI58, IRF9, OASL, MxB, UNC93B, CCR1, IFITM2, ISG54, RSAD2, IFI56, NMI, ADAR1, ZBP1, OAS2, ISG20, XAF1, C3aR, APOL2, CXCR4, IFI27, IFIT1, LYSP100, H28, STAT1, RIG-G, PML, USP18, GBP3, Ro52, Staf-50, GBP4, PL scramblase 1, GBP2, STAT1/STAT2, DDX60, ISG15, LGP2, TRIM5
Type I interferon signaling pathway	2.6913E-42	IRF7, SP100, IFI17, IFP 35, OAS1, IFI6, STAT2, OAS3, HLA-F, IFITM3, IRF9, OASL, MxB, IFITM2, ISG54, IFI56, ADAR1, OAS2, ISG20, XAF1, IFI27, STAT1, RIG-G, USP18, GBP2, STAT1/STAT2, ISG15

Genes with anti-HIV activity
Genes with antiviral activity (excluding HIV-1)
Genes with no direct antiviral activity or other functions

Note: p-value represents the probability that the genes are included in each pathway by chance

Supplementary Table 4. MetaCore process network analysis that depicts cellular and molecular processes characterizing the response to IFN-α in MT4R5 and SupT1R5R5.

Network	p-value	Genes from active data
Inflammation Interferon signaling	1.447E-26	IRF7, PKR, IFI17, TAP1 (PSF1), IFP 35, TLR3, IFI6, STAT2, GBP1, IRF9, MxB, IFITM2, ISG54, IFI56, ISG20, IFI27, STAT1, PML, IFI44, GBP2, STAT1/STAT2, ISG15
Immune response Innate immune response to RNA viral infection	1.797E-12	IRF7, MyD88, PKR, MDA-5, RIG-I, TLR3, STAT2, IRF9, ADAR1, STAT1
Inflammation Inflammasome	2.392E-05	IRF7, MyD88, PKR, MDA-5, RIG-I, TLR3, ISG15
Proliferation Negative regulation of cell proliferation	5.337E-04	TIG3, MyD88, PKR, IFI17, CCR1, STAT1, SSTR3
Immune response Antigen presentation	8.375E-04	Tapasin, PSME2, PSMB9, TAP1 (PSF1), STAT2, HLA-F, CEACAM1, STAT1
Inflammation IFN-γ signaling	5.164E-03	PKR, IFI16, IRF9, STAT1
Inflammation Jak-STAT Pathway	1.153E-02	STAT2, IL-15RA, CCR1, CXCR4, STAT1, STAT1/STAT2
Inflammation IL-12,15,18 signaling	2.210E-02	MyD88, STAT1, STAT1/STAT2
Development Regulation of angiogenesis	2.588E-02	CCR1, CEACAM1, CXCR4, STAT1
Development Blood vessel morphogenesis	2.846E-02	CCR1, CEACAM1, CXCR4, STAT1

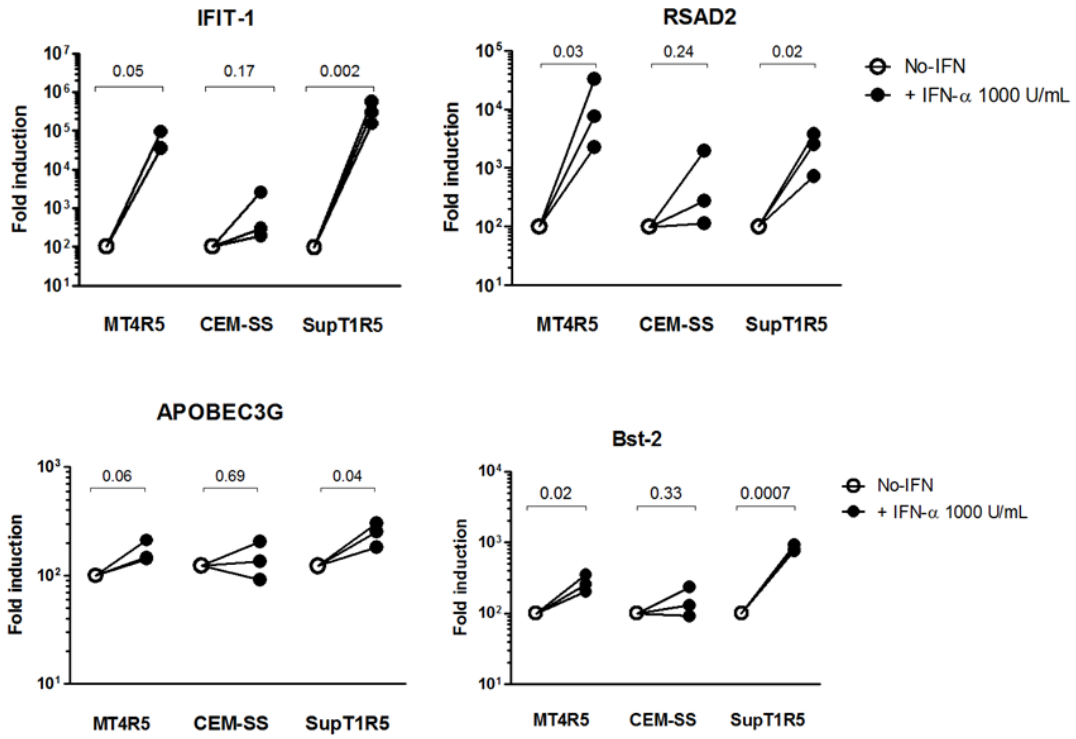
Genes with anti-HIV activity
Genes with antiviral activity (excluding HIV-1)
Genes with no direct antiviral activity or other functions

Note: p-value represents the probability that the genes are included in each pathway by chance

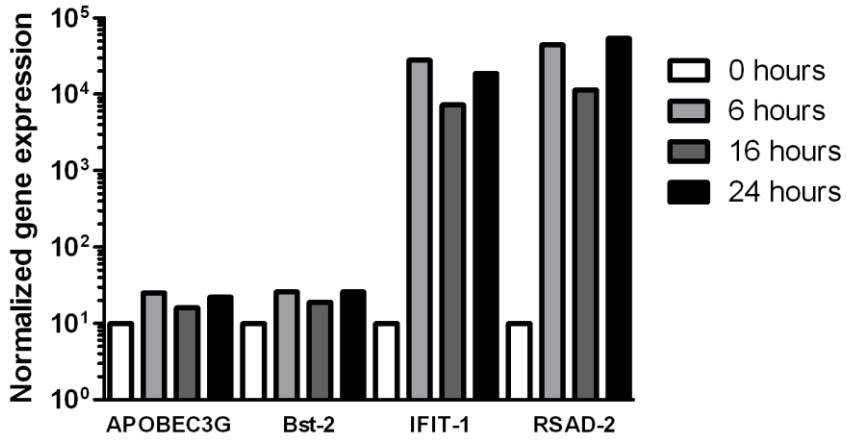
Supplementary Table 5. 44 genes upregulated by IFN-α in MT4R5 and SupT1R5R5 are frequently reported in the literature.

	Endogenous IFN-α production, *in vivo* HIV-1 infection					**Endogenous IFN-α production, *in vitro* HIV-1 infection**		**IFN-α treatment**
Gers-Huber et al. 2015	Bosinger et al. (PBMCs)[52]	Hyrcza et al. (Primary CD4+ T-cells)[6]	Rotger et al. (Primary CD4+ T-cells)[58]	Sedaghat et al. (Primary CD4+ T-cells)[59]	Smith et al. (Primary CD4+ T-cells)[60]	Audige et al. (Primary PBMCs)[7]	Woelk et al. (Primary MDM)[8]	Rempel et al. (Primary monocytes)[32]
IFIT1	x	x	x	x	x	x	x	x
IFIT3		x	x	x	x	x		x
OAS1	x	x	x	x	x		x	x
OAS2	x	x	x	x	x			x
STAT1	x	x	x	x	x	x	x	
IFI44		x	x	x	x	x	x	x
MX2	x	x	x		x	x	x	x
OASL		x	x	x	x	x		
IFITM1	x		x	x		x	x	x
ISG15	x		x	x	x		x	x
GBP1		x	x		x	x		
IFI35		x	x			x		
IFI44L		x	x	x	x	x		
IFI6			x	x	x	x		
OAS3	x	x	x	x				
IFI27		x	x	x		x		
IRF7		x	x	x		x		
PLSCR1		x					x	x
PSMB9		x	x					
TAP1			x			x		
BST2		x		x				
EIF2AK2		x				x		

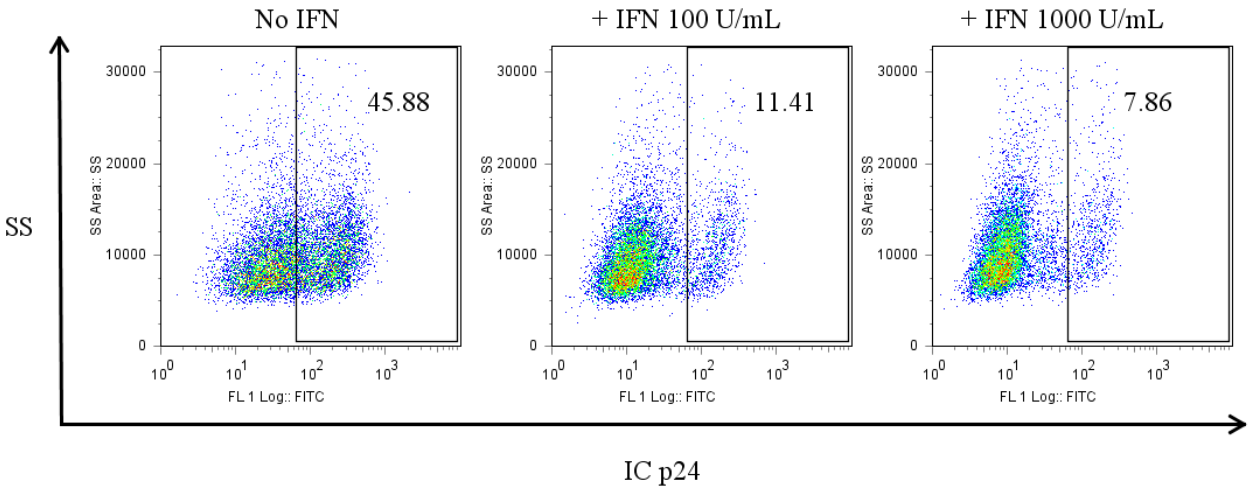
HERC5			x	x			x		
HERC6			x	x			x		
IFI16			x		x				
IFIT2							x		
ISG20	x					x	x		
LAP3			x	x			x		
LY6E			x	x			x		
RSAD2			x			x	x	x	
TRIM22			x	x					
USP18			x			x	x		
CD38			x	x					
DDX58			x				x		
IFIH1			x				x		
IFITM2				x					x
IFITM3			x	x				x	
IRF9									
LGALS3BP				x			x		
NMI									
SAMD9L				x		x	x		
SP110			x	x					
UBE2L6			x	x					
ZBP1			x				x		



Supplementary Figure 1. **The cell lines MT4R5 and SupT1R5 are responsive to IFN-α.** The capacity to upregulate ISGs was evaluated in the three cell lines MT4R5, CEM-SS and SupT1R5 treated with IFN-α at 1000 U/ml for 24h. Gene expression was then assessed by qPCR and normalized to that of GAPDH. Each circle represent an individual experiment (n=3). Statistics were performed using a paired Student's t-test. P-values are indicated for each condition.

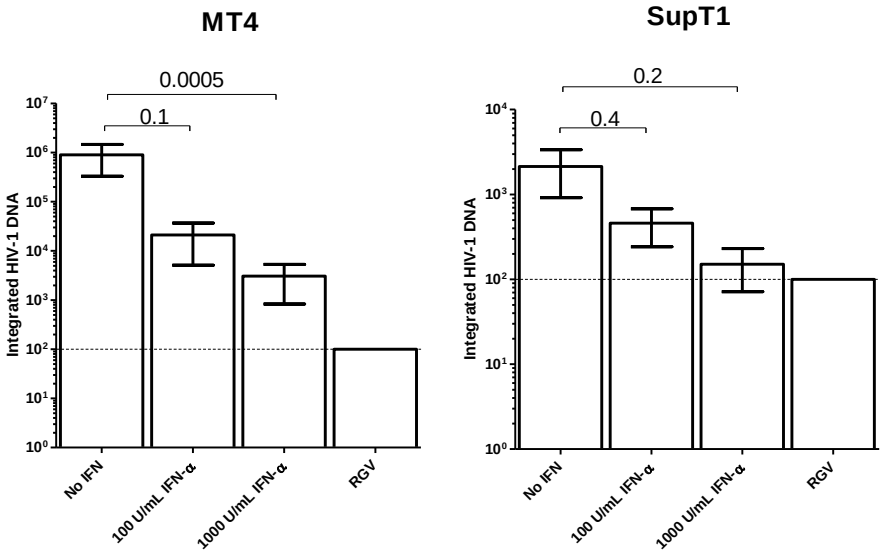


Supplementary Figure 2. Peak of ISG upregulation in MT4R5 cells is observed at 6 and 24 hours post IFN-treatment. Gene expression was assessed by qPCR and normalized to that of GAPDH for APOBEC3G, Bst-2, IFIT-1 and RSAD2. Cells were treated for 0, 6, 16 and 24 hours with 1000U/mL of IFN-α.

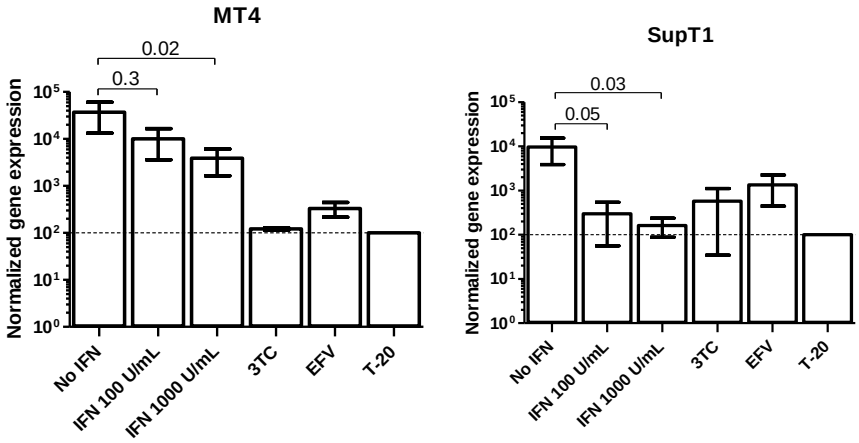


Supplementary Figure 3. HIV-1 protein production is strongly inhibited by IFN-α in MT4 cells. MT4 cells were treated with 0, 100 or 1000U/mL of IFN-α and subsequently infected with HIV-1 NL4-3. 48 hours post infection cells were stained for ic p24 expression. Data shown correspond to a single experiment representative of 3 independent experiments.

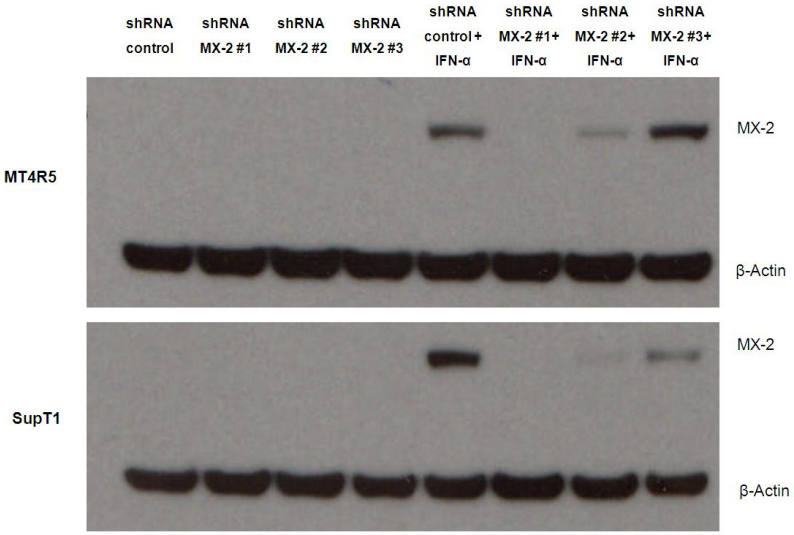
A B



C D



Supplementary Figure 4. **IFN-α treatment strongly inhibits HIV-1 integration and partially HIV-1 reverse transcription.** MT4 or SupT1 cells were treated 24 hours prior to HIV-1 infection with IFN-α (0, 100 or 1000U/mL) or treated with RGV 2 hours before infection. 48 hours post infection, cells were collected, lysed and integrated HIV-1 DNA was quantified using a two-step Alu qPCR. Integration of HIV-1 in MT4 (A) and SupT1 cells (B), normalized with the GAPDH cellular control. Levels of integration in RGV-treated cells are set to 100 as background of integration. Reverse transcription of HIV-1 in MT4 (C) and SupT1 cells (D), normalized with the GAPDH cellular control. T-20-treated cells are set to 100 as background of viral entry. The reverse transcriptase inhibitors (3TC and EFV) and the fusion inhibitors where considered as a positive control for inhibition of reverse transcription. Bars represent the average of three independent experiments. Error bars correspond to SEM. Statistics were performed using a paired Student's t-test. P-values are indicated for each condition.



Supplementary Figure 5. **MX2 is successfully downregulated in MT4R5 and SupT1R5 cells.** MX2 protein levels are depicted for MT4R5 cells (higher panel) and SupT1R5 cells (lower panel). β-actin was used as a loading control. The first 4 rows represent conditions without IFN-α treatment and the 4 last rows represent conditions with IFN-α treatment (1000U/mL).

3.2 Development of a humanized mouse model for HIV infection

3.2.1 Long-acting anti-retroviral drugs for treating HIV-1 in humanized mice

Despite the availability of very efficient antiretrovirals in the developed countries, HIV-1 pandemic increases every year. In order to generate novel treatments, it is necessary to increase our knowledge about viral replication, latency, antiretroviral treatment (ART), not only *in vitro* but also *in vivo*. We made use of the humanized mice as an *in vivo* model for studying HIV-1 replication, ART and we also showed that latency can be studied with this model. The results of these experiments were published in the manuscript below.

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Own contribution:

I assisted Marc Nischang for all experiments *in vivo* (humanized mouse), including the isolation of CD34+ cells from cord blood, the transplantation of the newborn mice with CD34+ cells, bleeding of the mice, periodic health check of the mice, euthanasia of the mice and processing of the organs.

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PLoS one

Humanized Mice Recapitulate Key Features of HIV-1 Infection: A Novel Concept Using Long-Acting Anti-Retroviral Drugs for Treating HIV-1

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Abstract

Background: Humanized mice generate a lymphoid system of human origin subsequent to transplantation of human CD34+ cells and thus are highly susceptible to HIV infection. Here we examined the efficacy of antiretroviral treatment (ART) when added to food pellets, and of long-acting (LA) antiretroviral compounds, either as monotherapy or in combination. These studies shall be inspiring for establishing a gold standard of ART, which is easy to administer and well supported by the mice, and for subsequent studies such as latency. Furthermore, they should disclose whether viral breakthrough and emergence of resistance occurs similar as in HIV-infected patients when ART is insufficient.

Methods/Principal Findings: NOD/shi-scid/$\gamma_c$null (NOG) mice were used in all experimentations. We first performed pharmacokinetic studies of the drugs used, either added to food pellets (AZT, TDF, 3TC, RTV) or in a LA formulation that permitted once weekly subcutaneous administration (TMC278: non-nucleoside reverse transcriptase inhibitor, TMC181: protease inhibitor). A combination of 3TC, TDF and TMC278-LA or 3TC, TDF, TMC278-LA and TMC181-LA suppressed the viral load to undetectable levels in 15/19 (79%) and 14/14 (100%) mice, respectively. In successfully treated mice, subsequent monotherapy with TMC278-LA resulted in viral breakthrough; in contrast, the two LA compounds together prevented viral breakthrough. Resistance mutations matched the mutations most commonly observed in HIV patients failing therapy. Importantly, viral rebound after interruption of ART, presence of HIV DNA in successfully treated mice and in vitro reactivation of early HIV transcripts point to an existing latent HIV reservoir.

Conclusions/Significance: This report is a unique description of multiple aspects of HIV infection in humanized mice that comprised efficacy testing of various treatment regimens, including LA compounds, resistance mutation analysis as well as viral rebound after treatment interruption. Humanized mice will be highly valuable for exploring the antiviral potency of new compounds or compounds targeting the latent HIV reservoir.

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Competing Interests: The following authors are employees from Tibotec BVBA, Belgium: KA, BS, AvC, GK, DB and RS. The laboratory from RFS has been financially supported by Tibotec BVBA; in addition, Tibotec BVBA provided reagents to the laboratory of RFS and performed a number of assays in the context of the collaboration between the laboratory of RFS and Tibotec BVBA. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data materials.

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Introduction

The HIV pandemic continues to spread. Even in the United States and Europe with their relative universal access to combined anti-retroviral treatment (ART), the prevalence of HIV-infected people is increasing. The 2nd and 3rd generation antivirals are very efficacious. The life expectancy of treated HIV-infected individuals has significantly improved over the last two decades [1] and, in turn, has contributed to the increasing prevalence. However, ART has significant shortcomings, including adverse events, psychological dependence, life-long adherence and cost. Incomplete adherence results in the emergence of drug-resistant HIV strains. Novel and simpler treatment strategies and, in the best-case scenario, a cure are needed.

The HIV pandemic started with an ancestral SIV from a non-human primate crossing into humans [2], and thus, it is not surprising that HIV replication is limited to human and non-human primate cells. Mouse models of HIV infection have been generated by engrafting human lymphoid tissue into SCID mice [3] and are receptive to HIV [4]. For example, SCID mice

transplanted with fetal liver (liv) and thymus (thy) tissue were very valuable for studying various aspects of HIV pathogenesis, including HIV-induced pathology in the thy/liv implant, screening anti-viral compounds and hematopoietic stem cell–based gene therapy [5]. However, these studies are limited to the thy/liv implant.

Development of a human adaptive immune system in cord blood cell–transplanted mice [6] renewed the interest in humanized mice (hu mice): hematopoietic CD34+ cells were preferentially transplanted into the liver of newborn mice or i.v. at older age. The mice develop a lymphoid-like system of human origin with T and B cells, monocytes, plasmacytoid and conventional DCs, thymus and lymph nodes [7]. Their mature T cells have a broad Vβ repertoire, and more than 40% of T cells display a naive phenotype [6]. This breakthrough was only realized through the development of heavily immunodeficient mice by crossing of SCID mice with non-obese diabetic (NOD) mice and mice deficient in the gamma c (γ_c) chain of the IL-2 receptor or the generation of Rag1 or $2-/-$ $\gamma_c-/-$ knock-out mice [8].

We and others demonstrated that these hu mice are highly permissive to HIV infection when challenged with CCR5- or CXCR4-tropic HIV strains and show viral dissemination and progressive CD4+ T-cell loss [9,10,11,12,13].

To validate the experimental significance of hu mice for studying HIV pathogenesis and their value for novel interventional approaches, key aspects of HIV infection/pathogenesis must be fulfilled. The model should recapitulate ART of disseminated HIV infection with subsequent recovery of the immune system; interruption of ART should result in a rebound of HIV replication from the latent reservoir. Five studies reported the effects of ART in HIV-infected humanized mice using different drugs and drug combinations [14,15,16,17,18]. While these reports are very promising that humanized mice may be the long awaited small animal model for preclinical proof-of-concept studies, they lack pharmacokinetic (PK) studies for the medicinal compounds used except for the report by Choudhary et al [15] that would help to compare the data obtained in humanized mice to a clinical context.

A "standardized" ART scheme that completely suppresses HIV RNA replication in hu mice would be highly valuable for preclinical proof-of-concept studies for novel anti-retroviral compounds and studies of latency that closely approximate the situation in HIV-infected humans treated with ART.

Thus, we sought to determine if this mouse model is valuable for studying antiretroviral treatment of disseminated HIV infection and if it recapitulates key features, such as viral rebound, breakthrough replication and viral rebound subsequent to interruption of ART. We made a major effort to define the dose of antiretroviral compounds added to the food pellets to compare data to human studies. We also benefited from access to antiretroviral compounds in special formulation that permit once weekly dosing in mice. Thus, we also were able to study a novel concept of anti-HIV therapy based on LA antiretroviral drugs.

Results

Engraftment with human cells at around week 15 when hu mice were infected with HIV

Newborn NOG mice were transplanted with CD34+ haematopoietic progenitor cells isolated from umbilical cord blood. At around 15 weeks of age, the engraftment level was 20.7%±13.2 (avg ± std) before HIV infection (Figure S1). Of all human cells, CD4+ T cells were 23.1%±14.5 (avg ± std), CD8+ T cells 12.9%±9, and CD19+ B cells 50.8%±24.2. These engraftment values and their cell subset distributions are similar as reported previously [11,19,20].

Pharmacokinetics of 3TC, Tenofovir (TDF), TMC278-long-acting (LA) and TMC181-LA

The easiest and most convenient way for long-term ART in hu mice would be to add the ART to the food pellets. Mice have a higher metabolism than humans, and thus, we converted the dose of the distinct compounds used in humans by a formula as described [21]. The dose calculated was 61.7 mg/kg/day for 3TC and for TDF, considering food uptake of 3–4 g/d for a hu mouse with a body weight of 20–30 g. We generated food pellets containing 0.5 mg of 3TC and TDF per g of food. 3TC and TDF belong to the group of nucleoside resp. nucleotide reverse transcriptase inhibitors (NRTIs).

PK data validated this approach showing plasma levels in the therapeutic range over the entire observation period with fluctuations due to the wake-sleep cycle of the mice (Fig. 1A–D). In contrast, azidothymidine (AZT) at 0.5 mg/g of food and ritonavir (RTV) at 1 mg/g of food gave toxic concentrations clearly above the therapeutic range or sub-inhibitory concentrations, respectively (Figure S2). The plasma concentration of TMC278-LA and TMC181-LA (at the higher dose) was still clearly above the target concentration (C_{target}) even 14 days after s.c. injection (Fig. 1E–F). TMC278 is the recently approved non-nucleoside reverse transcriptase inhibitor (NNRTI) rilpivirine [22], and TMC181 is a pre-clinical-stage protease inhibitor (PI) belonging to the same chemical class as TMC114 (darunavir), but displaying better potency while preserving similarly high resistance coverage. The PK data of the long-acting drugs permitted a once weekly application in mice.

HIV RNA plasma level is suppressed by ART and promptly rebounds with treatment interruption

In a pilot experiment, uninfected mice appeared to tolerate AZT, 3TC and RTV well. Thus, while awaiting the PK data of these compounds, we started a first experiment to examine ART in HIV-infected mice (Fig. 2). Mice before ART had an HIV RNA baseline of $10^{4.9}\pm10^{5.2}$ copies/ml (avg ± std). Unexpectedly, the treated mice developed wasting within 2 weeks, which we attributed to the ART and, in particular, AZT. We therefore changed the ART immediately to TDF, 3TC and TMC278-LA, and within 1 week, the mice recovered from the wasting disease. Importantly, within 4–8 weeks, 14/21 (66%) mice showed a decline of HIV RNA levels to under the detection limit of 800 copies/ml (Fig. 2A). One mouse with detectable HIV RNA at that time showed suppressed HIV RNA when we bled it 72 days after start of ART (Fig. 2A). Since we switched the ART in two mice with detectable HIV RNA to monotherapy with TMC278-LA, we are not able to make any statement about their eventual response rate if the ART had been continued. Thus, the overall response rate to the ART (3TC/TDF/TMC278-LA) was 79% (15/19 mice).

In an additional group of mice treated with AZT, 3TC and RTV, we found various laboratory disturbances, most prominent a very significant anemia (Figure S3). In concert with plasma AZT levels clearly above the therapeutic range, we attributed the wasting observed to AZT toxicity.

Nearly all mice receiving the 2 weeks of AZT, 3TC and RTV treatment suffered from weight loss (Fig. 2E and F). Remarkably, the mice with viral failure experienced more significant weight loss than the others (Fig. 2G). Mock-treated HIV-infected mice showed

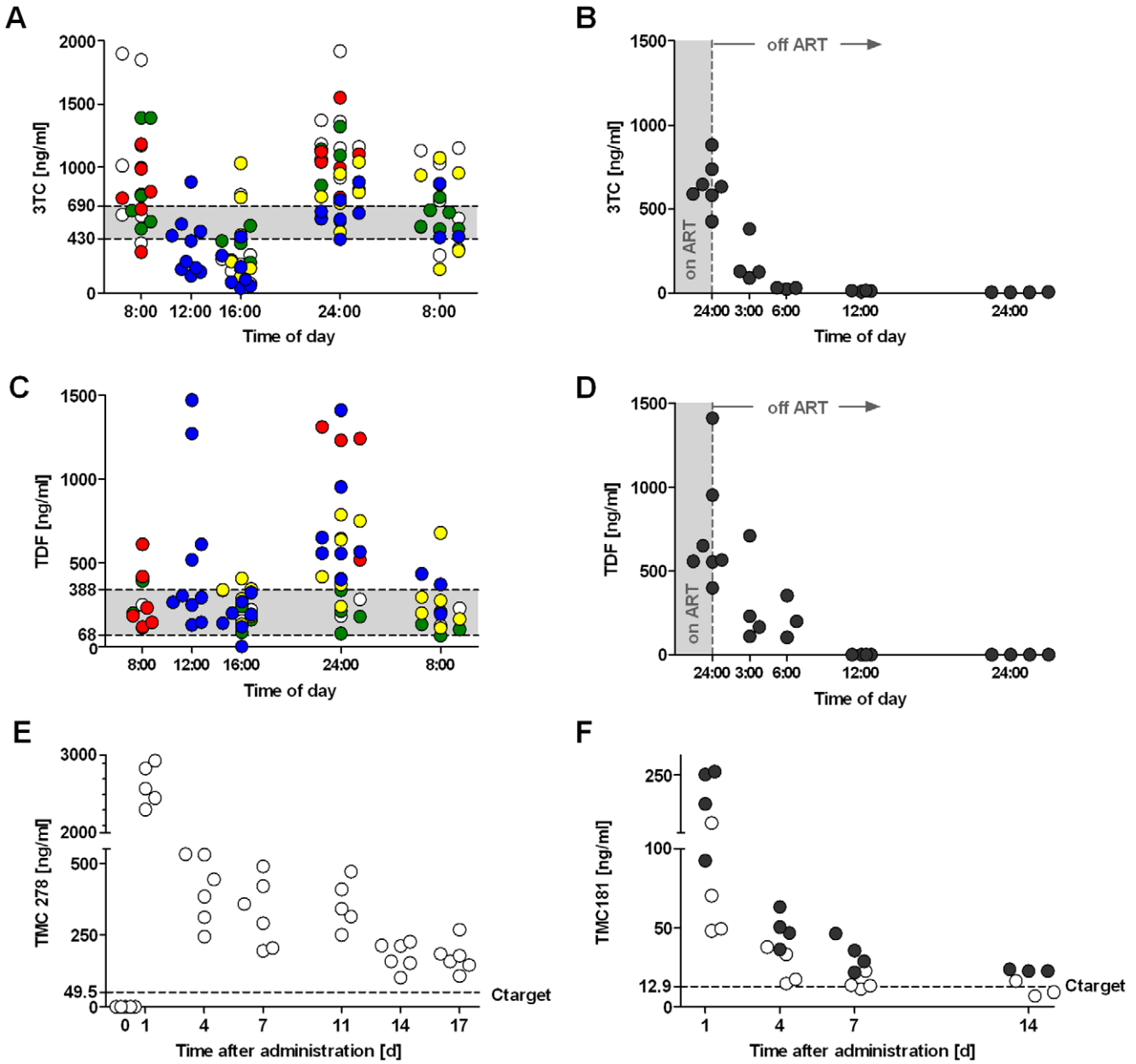

Figure 1. PK data for 3TC, TDF, TMC278-LA and TMC181-LA. (A and C) Plasma levels of 3TC and TDF, respectively, over a day of mice on food pellets containing 0.5 mg/g food of 3TC or TDF for 2 weeks. (B and D) Decay rate of 3TC ($t_{1/2} = 5.5$ h) and TDF ($t_{1/2} = 3.5$ h), respectively, when replacing the food containing 3TC or TDF with standard food. (E and F) Plasma levels after one dose of either TMC278-LA (160 mg/kg) or TMC181-LA (white dots: 200mg/kg; black dots: 400mg/kg) administered s.c. The data were obtained with mice on ART-containing food pellets for at least 2 weeks to permit PK equilibration. The shaded area in (A–D) indicates the therapeutic range as defined in humans [42,43]. The dashed line in (E and F) indicates the target concentration (C target). Median effective concentration (EC) 50 values of TMC278 and TMC181 are 4.95 ng/ml and 1.29 ng/ml respectively *in vitro* in MT4 cells cultured with 50% human serum. The different colours indicate the experiments done with the same food batch, and whether we used mice transplanted with human CD34+ cells or not (White, red and yellow dots indicate humanized mice, green and blue dots indicate mice without transplantation of human CD34+ cells).
doi:10.1371/journal.pone.0038853.g001

a very stable weight course with fluctuations of less than 2 g over time.

We subsequently divided the mice with suppressed HIV RNA into two groups, seven mice were maintained on ART for another 5 weeks and, thereafter, treatment was interrupted. The other seven mice were treated by TMC278-LA alone. ART interruption resulted in viral rebound in all mice, indicating the existence of a latent reservoir, such as that in HIV-infected humans (Fig. 2C). Monotherapy with TMC278-LA was insufficiently potent to suppress HIV RNA since 6/7 mice showed a breakthrough of viral replication (Fig. 2D).

While there were no obvious symptoms or signs, we observed a higher mortality in mock-treated HIV infected mice than in ART treated mice. We associated this higher mortality with an unknown HIV-associated phenomenon. This mortality was usually less than 20% and thus when working with small numbers of mice, there

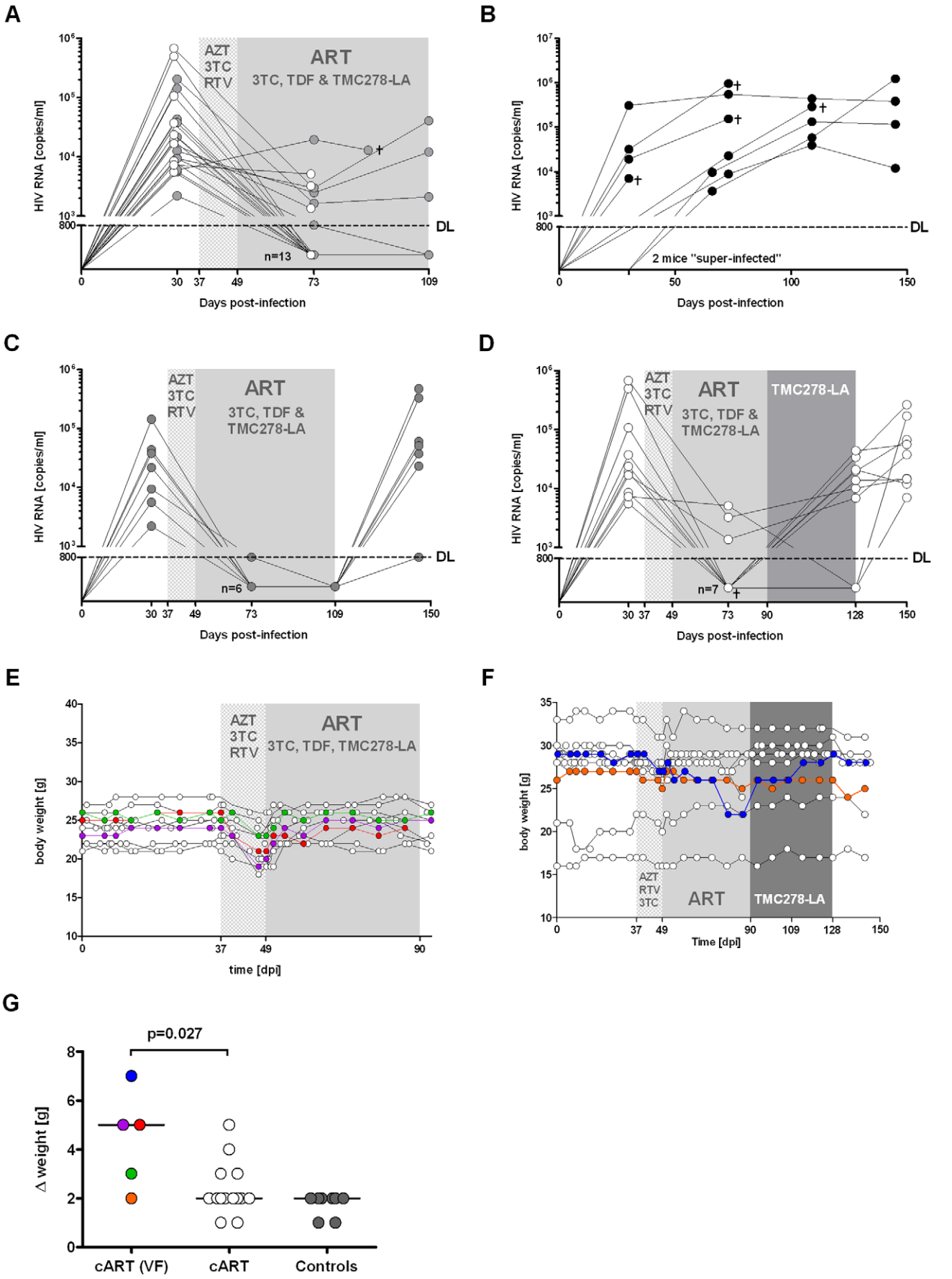
A
HIV RNA [copies/ml]
AZT
3TC
RTV
ART
3TC, TDF & TMC278-LA
DL
n=13
Days post-infection
B
HIV RNA [copies/ml]
DL
2 mice "super-infected"
Days post-infection
C
HIV RNA [copies/ml]
AZT
3TC
RTV
ART
3TC, TDF &
TMC278-LA
DL
n=6
Days post-infection
D
HIV RNA [copies/ml]
AZT
3TC
RTV
ART
3TC, TDF &
TMC278-LA
TMC278-LA
DL
n=7
Days post-infection
E
body weight [g]
AZT
3TC
RTV
ART
3TC, TDF, TMC278-LA
time [dpi]
F
body weight [g]
AZT
RTV
3TC
ART
TMC278-LA
Time [dpi]
G
p=0.027
Δ weight [g]
cART (VF)
cART
Controls

Figure 2. ART is highly efficient in disseminated high-titer HIV infection in hu mice. (A) Response to ART with AZT, 3TC and RTV, followed by 3TC, TDF and TMC278-LA. Note that the initial regimen was poorly tolerated by the hu mice that resulted in reduced food uptake and thus a somewhat lower response rate. (B) Mock-treated HIV-infected mice (n = 8). (C and D) Mice with suppressed HIV RNA were either kept on ART (n = 11) (C) or switched to monotherapy with TMC278-LA alone (n = 10) (D). ART and monotherapy were interrupted to monitor the mice for viral rebound. We included two mice with viral failure into the group treated with TMC-278-LA alone. † indicates that one mouse died within one week of bleeding. (E and F) Weight monitoring of mice either constantly on combined ART (E) or switched subsequently to monotherapy with TMC278-LA (F). Compilation of weight loss over time of all mice, i.e., mice on ART with viral failure (VF), mice on ART with suppressed HIV RNA and untreated HIV-infected mice (controls) (G). Grey-spotted area indicates the time period hu mice were on ART with AZT, 3TC and RTV, grey-plain shaded the time period on ART with 3TC, TDF and TMC278-LA. The coloured circles indicate the mice with viral failure.
doi:10.1371/journal.pone.0038853.g002

may be divergence from this estimated mortality rate. In the 2nd set of experiments presented (see below), we had no loss due to "spontaneous" mortality.

Emergence of drug-resistant HIV strains in hu mice

All mice on ART with 3TC, TDF and TMC278-LA which experienced viral failure revealed the consecutive or simultaneous emergence of the prototype 3TC mutation M184I and the TMC278 mutation E138K (Fig. 3 and Table 1). All but one mouse showed viral failure when treated with TMC278-LA monotherapy. Of those mice with viral failure, all but one had the E138K mutation either alone or with the M184I mutation (Table 1). It is unknown if minor drug variants or mutations outside the amplified RT region might explain the lack of any TMC278 resistance mutation detected in the one mouse scored fully susceptible to TMC278 [23].

ART with two long-acting drugs (TMC278-LA and TMC181-LA) effectively treats HIV-infected hu-mice

Next, we sought to determine if a simplified ART of two long-acting drugs interfering at different steps in the HIV replication cycle is effective as maintenance therapy to keep HIV RNA under the detection limit. Initial ART was with TDF, 3TC, TMC278-LA and TMC181-LA. In mice with documented HIV RNA below detection limit after 44 days of ART, we simplified the regimen as planned. The other mice that still showed a marked viremia were continued on the quadruple ART. The dual ART consisting of TMC278-LA and TMC181-LA given once weekly s.c. was really potent: all but one mouse (viral blip) had undetectable HIV RNA (Fig. 4B). Note, that the HIV RNA of all mice which were maintained on the quadruple ART became negative at 99 days after start of treatment (Fig. 4A). This variable decay rate in response to ART mirrors the distinct decay rate of HIV in patients

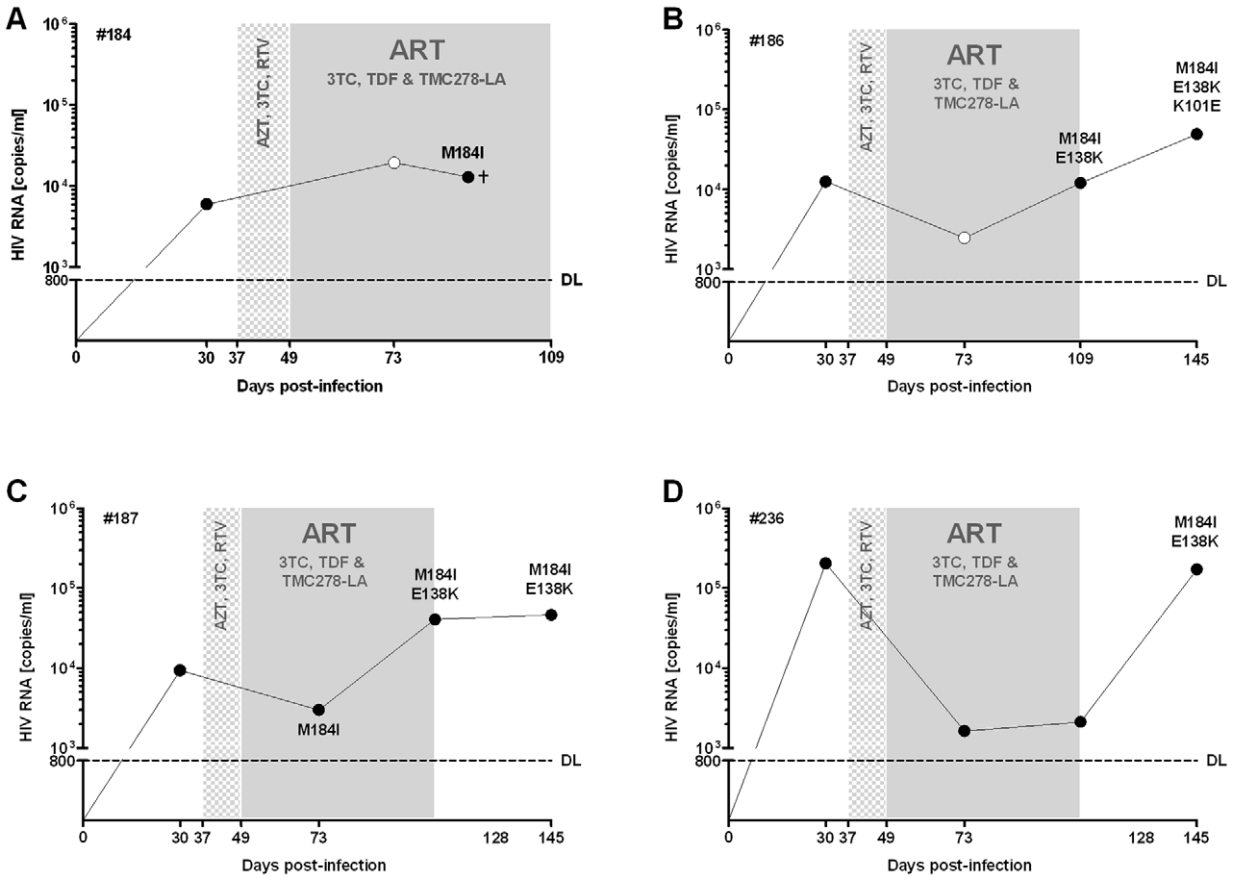

Figure 3. Humanized mice with viral failure under ART with 3TC, TDF and TMC278-LA show consecutive or simultaneous emergence of the key mutation to 3TC or TMC278. A), B), C) and D) show the four mice with emergence of resistance under ART. The black dots indicate that resistance testing had been done, white dots no resistance testing done.
doi:10.1371/journal.pone.0038853.g003

Table 1. Emergence of resistance in the mice under monotherapy with TMC278-LA.

Mouse (Identifier)	day 30 (baseline)	day 73 (under ART with 3TC, TDF, TMC278-LA)	day 128 (38 days under TMC278-LA alone)	day 150 (22 days after interruption of TMC278-LA)
# 192**	S*	n.d.¶	S	S
# 242	S	n.d.	n.d.	S
# 232	S	n.d.	E138K	E138K
# 190***	S	M184V	n.d.	M184I/E138K
# 189	S	S	M184I/K101E/E138K	M184I/K101E/E138K
# 191***	S	M184I	M184I/E138K	M184I/E138K
# 224***	S	M184I	n.d.	M184I/E138K

*S = susceptible (wildtype strain).
**#192 showed suppressed HIV RNA under TMC278-LA monotherapy.
***#191, 224 showed viral failure under the ART regimen of 3TC, TDF and TMC278-LA. #190 gave a positive signal for HIV RNA but below the limit of detection (<800 copies/ml)
#221, 245 only baseline analyses have been done, and therefore data from these mice were not integrated in the table.
¶#n.d. = not done.
doi:10.1371/journal.pone.0038853.t001

starting ART. As expected the CD4/CD8 T-cell ratio was significantly higher in the mice with suppressed HIV RNA than in control mice (Fig. 4D).

ART-treated HIV-infected hu mice had HIV RNA under 50 copies/ml but detectable cell-associated HIV DNA

The limited amounts of blood in any running experiment held the sensitivity of the Amplicor Roche® to 400–800 copies/ml. Although it is unlikely, this detection limit does not exclude low viral replication in the ART treated mice. The HIV RNA measurement based on the final bleeding of the mice treated either with the dual ART or the quadruple ART (3TC, TDF, TMC-278LA, TMC181-LA) revealed that 8/10 mice had fewer than 60 copies/ml (Table 2), which emphasizes the efficacy of the ART in the current setting.

In the mice from the second experiment (i.e., mice as shown in Fig. 4), we determined if cell-associated HIV DNA was detectable in splenocytes. Using a real-time PCR specific for YU-2, this was indeed the case in 13/15 mice (Fig. 5A); in all untreated mice, we detected levels of cell-associated HIV DNA higher than in treated ones with the exception of mouse #417. This mouse showed a low-level viremia despite ART that easily explains the relatively high cell-associated HIV DNA. We observed no correlation between cell-associated HIV DNA and engraftment levels or peak viremia.

In vitro reactivation of HIV transcripts in spleen specimens from hu mice in response to re-activating compounds

In order to examine whether latently infected cells exist in hu mice, we examined unspliced HIV Gag mRNA transcripts in spleen specimens from HIV-infected mock- and vero-treated mice prior and after *in vitro* reactivation. Since we were limited in spleen specimens, we opted for a combination of mitogens in concert with anti-CD3/CD28 and IL-7 to increase our chances of successful reactivation. The short term assay we used as well as the anti-apoptotic effect of IL-7 was certainly beneficial for counteracting any toxic effects due to the application of such a cocktail. Indeed, we did not observe any toxic effects 18 h after adding this cocktail when we harvested the tissue for quantifying HIV mRNA transcripts. As previously reported [16,17,18], we observed a clear reactivation of HIV Gag transcripts after stimulation (Fig. 5B). We did not isolate the human CD4+ cells to perform *in vitro* reactivation studies – however, we have no reason to assume that the cell subset harbouring latently HIV will differ from the results published [16,17,18].

Discussion

We sought to determine if hu mice recapitulate key features of HIV infection and treatment and to assess the value of long-acting anti-HIV drugs for treating HIV infection. We found that i) conventional ART with two NRTIs and a NNRTI efficiently suppressed HIV viral load and allowed recovery of the immune system; ii) cell-associated HIV DNA was still present in those mice, and interruption of ART resulted in viral rebound; *in vitro* reactivation of spleen specimens from successfully treated mice yielded increased number of HIV mRNA transcripts as compared to baseline; and iii) simplification of ART with two long-acting drugs kept HIV RNA suppressed. Thus, HIV infection in hu mice mirrors key features of HIV infection in humans, including high titer viremia in untreated mice, suppression of HIV RNA when treated with ART but emergence of resistance when treated with insufficient regimens, viral rebound after treatment interruption, and recovery of CD4+ T cells under ART. Thus, hu mice are a highly valuable animal model to assess the antiviral potency of new compounds or novel strategies to eradicate latent HIV.

Liv/thy SCID hu mice have been used before to investigate novel compounds for their anti-HIV activity [5,24]. However, detailed PK data were mostly lacking, thus making it difficult to interpret the antiviral potency of the compounds. Furthermore, conventional liv/thy SCID hu mice lack peripheral T cells [3]. Thus, SCID-hu Thy/Liv mice were rarely used to study compounds to treat disseminated HIV infection. Most studies administered the drugs either before or immediately after the HIV challenge [25], and that recapitulates post- or pre-exposure prophylaxis where less potent regimens are already effective.

The new generation of hu mice displays an elaborated lymphoid-like system of human origin [6] and is highly susceptible to HIV infection. However, studies with these mice lacked data about their value for standardized pre-clinical testing. Five studies [14,15,16,17,18] reported the efficacy of ART in HIV-infected mice. In these studies, ART was started within 3 weeks after HIV

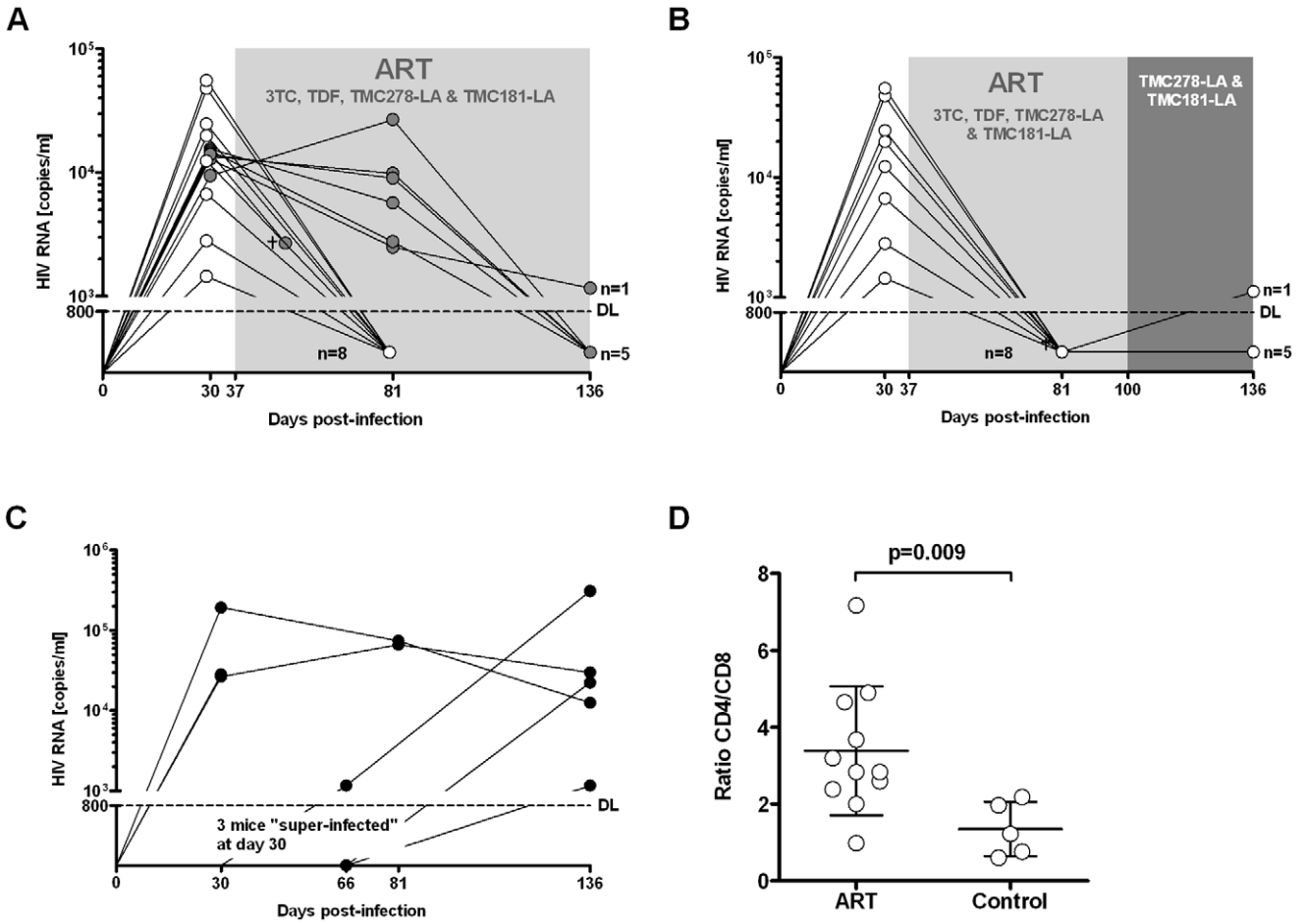

Figure 4. Two long-acting drugs, TMC278-LA (160 mg/kg) and TMC181-LA (400 mg/kg), are highly effective as maintenance therapy. (A) Response to a quadruple ART consisting of 3TC, TDF, TMC278-LA and TMC181-LA over a treatment period of 150 days (the black circles identifies the mice which remained over the entire time on ART (n = 7), the white circles identify the mice which were subsequently switched to a treatment with double long-acting drugs (see (B)). (B) Sustained successful suppression of HIV RNA after switching mice with suppressed HIV RNA under quadruple ART to a treatment with double long-acting drugs (n = 8). (C) Mock-treated HIV infected mice (n = 5). (D) CD4+ T-cells as determined by the CD4/CD8 cell ratio in all treated mice (ART and double long-acting drugs) and mock-treated mice at the end of the experiment.
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infection (i.e., rather early after HIV challenge). In the first study, HIV was suppressed in 3/6 mice by i.p. ART, and HIV recurred when treatment was interrupted [15]. Surprisingly, a second study did not observe viral rebound after treatment interruption [14]. In subsequent studies, complete response rates were reported with an intensified regimen (i.p. administration of emtricitabine (FTC), TDF, an integrase inhibitor and enfuvirtide) [16] or high doses of ART given i.p [17]. All but one [16] of these studies lack detailed PK data on the administered drugs and data related to long-term administration of ART and to the anti-HIV efficacy of ART in chronically infected mice. Compared to the PK data we generated in mice and considering the therapeutic range in humans, the dosages applied in some of the studies reported are most likely 3–5-fold over the therapeutic range.

For an effective mouse model, long-term ART must be non-toxic and well tolerated by the mice. This requires solid PK data in

Table 2. HIV RNA load at the terminal bleeding in mice on ART or on double long-acting drugs.

	Mice on quadruple ART				Mice on double-long acting drugs					
Mouse identification number	#411	#412	#417	#432	#459	#402	#413	#415	#457	#466
HIV RNA	n.d.*	n.d.	152	n.d.	<40	<60	502	n.d.	n.d.	n.d.
Detection limit [copies/ml]	40	40	40	40	40	60	40	60	60	40

*n.d. = non-detectable.
- Humanized mice were sacrificed 151 days after HIV infection and 114 days after starting ART or double long-acting drugs.
- Detection limit: the volume of plasma available was slightly different for the mice euthanized and thus the lower detection limit varied accordingly between 40 and 60 copies/ml.
doi:10.1371/journal.pone.0038853.t002

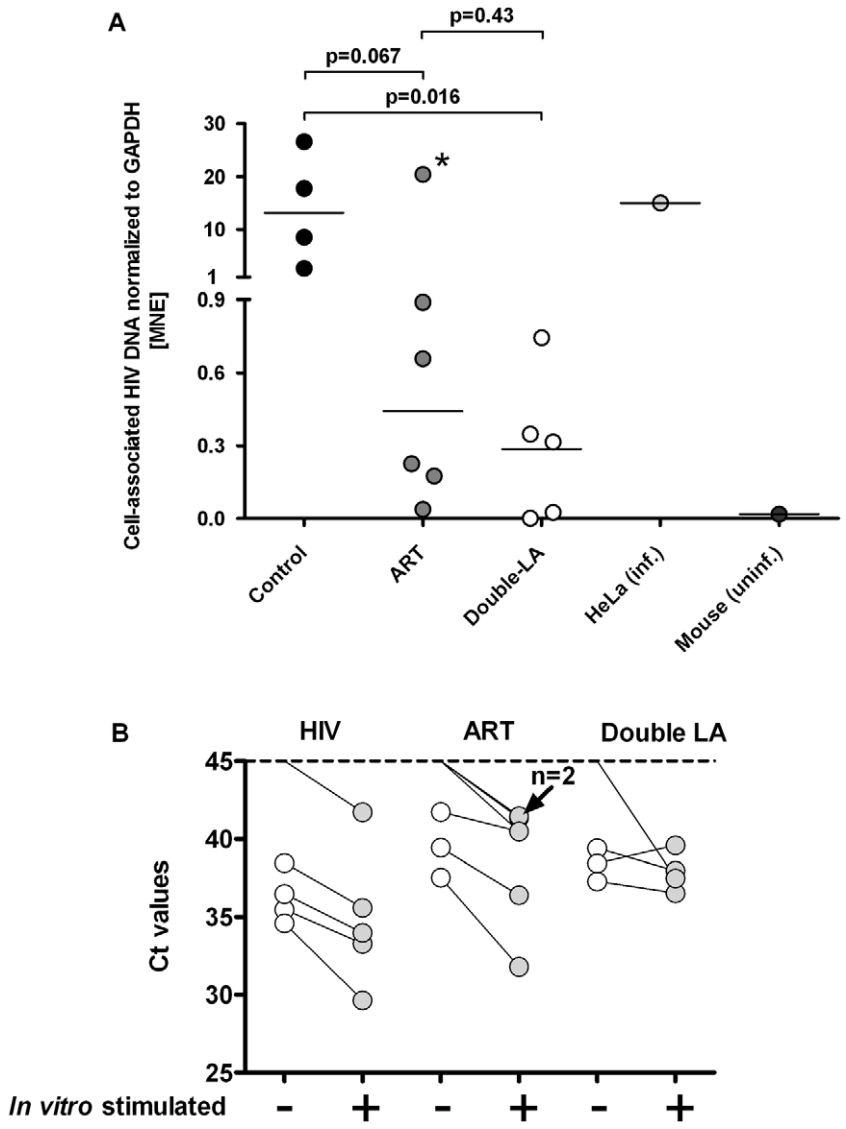

Figure 5. Recovery of cell-associated HIV DNA (A) and increase of HIV mRNA transcripts in vitro from splenic tissue obtained from HIV-infected mice with suppressed HIV RNA following activation. (A) DNA from infected HeLa cells (HeLa inf) and from the spleen of HIV-infected mice served as positive controls, DNA from an uninfected humanized mouse (uninf) served as negative control. The specimens of the treated and HIV-infected mice were from the experiments investigating the antiviral potency of the double long-acting drugs; (MNE = mean normalized expression). B) Splenic tissue specimens from either HIV infected ART naïve hu mice (HIV), ART treated mice (ART) or mice treated with the two long acting drugs (Double-LA) were subjected to mitogens (PMA, PHA) in concert with anti-CD3/28 and IL-7. 18 hours later RNA was extracted and real-time PCR done for quantifying HIV Gag transcripts. Specimens of two mice which were treated with double LA drugs did not show any HIV transcript at all (data not shown in the graph); in five mice we did not detect any HIV transcripts prior to stimulation. The real-time PCRs were done in duplicates. *this specimen is from a mouse (#417) with detectable HIV RNA at the time of euthanization (see Table 2).
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the therapeutic range for humans. In our experiments, we added the distinct anti-HIV compounds to the food pellet based upon calculating food intake, weight and metabolic rate. We generated PK data in the therapeutic range for 3TC and TDF. The long-acting drugs TMC278-LA and TMC181-LA were injected s.c. once weekly. We identified dosages that resulted in concentrations clearly above the C_{target} 1 week after its administration. Notably, plasma levels of NRTIs approximate only the concentration of the anti-HIV active intracellular tri-phosphorylated compounds. Since the half-life of the active moiety is longer than from the parental compound [26], we were confident that the dosages would be efficacious for treating disseminated HIV infection in our model. Indeed, this was the case. We observed HIV RNA suppression in 79% in the first and 100% in the second experiment.

From the PK data, the mice in the first experiment were treated for the first 2 weeks with only effective dosages of 3TC and AZT, a dual therapy insufficient to suppress HIV; RTV plasma levels were substantially below the therapeutic range. Furthermore, AZT which was toxic for the mice resulted in decreased food uptake that was reflected by substantial weight loss and sub-therapeutic dose levels. The emergence of drug-resistant HIV was a logical consequence of insufficient ART plasma levels. The observed M184I mutations in our study are the most prevalent among TDF/FTC-treated HIV individuals. Furthermore, we selected the TMC278 E138K resistance–associated mutation in our mouse

model. These findings demonstrate that our model consistently reproduced the Phase III trial results of the ECHO & THRIVE studies, which showed that E138K and M184I combination was the most observed resistance associated mutations in patients treated with TMC278 and co-formulated TDF/FTC [27,28]. Indeed, our data indicate that the M184I mutation precedes the E138K mutation; this suggests that patients with archived HIV strains with the M184I are especially prone to viral failure with a subsequent triple compound–based regimen with 3TC or FTC and TMC278. This mimics what happens in patients with poor adherence and emphasizes the preclinical proof-of concept value of this mouse model for HIV infection.

HIV-infected mice displayed a distinct response rate to ART (i.e., around 50% of all mice under quadruple therapy had an undetectable HIV RNA at day 44, the other 50% at day 99) (Fig. 4A). Since we were limited in blood draws, we have no detailed data about the viral decay in these sets of mice. Notably, we observed a rapid drop of HIV RNA in a majority of HIV-infected mice within 10 days after initiation of ART when we took blood in short intervals (unpublished data). In any case, the response rate to ART may vary in HIV-infected hu mice similar to the variable response observed in HIV-infected individuals starting ART. It would be therefore sensible to perform HIV RNA measurements over at least 2 months to document ART response or failure.

In our experiments, blood draws yielded small amounts, but terminal bleeds yields larger blood volumes, which are a critical determinant for the sensitivity of the Amplicor Roche®. Indeed, the larger blood volumes documented the success of ART with HIV RNA copy numbers below 60/ml in most mice, thus excluding that low-level viremia was still ongoing in the successfully treated animals.

Interrupting ART resulted in prompt viral rebound in all mice. The mice used for that purpose successfully responded to ART and were treated for a longer period of time to assure the decay of potentially low-replicating cells. We also found cell-associated HIV proviral DNA in untreated and successfully treated mice, re-enforcing the fact that HIV generates a latent reservoir in HIV-infected hu mice. Besides, cell-associated HIV proviral DNA levels in untreated mice were higher than in treated ones, similar to the case in humans [29]. Similarly to findings reported [16,17,18], we observed a clear increase of HIV transcripts when splenic tissue from HIV infected mice with suppressed HIV RNA was stimulated. These findings are promising for using hu mice for studying the latent reservoir and in particular for studying approaches to eradicate it. We observed no correlation between cellular HIV-associated proviral DNA and engraftment level or peak viremia. These data show that hu mice represent a model to study latency and potentially novel treatment strategies. Viral rebound or its absence *in vivo* will be a definitive end point of novel eradication strategies aiming to cure HIV infection.

We also report the use of our HIV hu mouse model in the study of TMC278 and TMC181 in a galenic formulation that results in plasma levels above the C_{target} for at least 1 week after administration (long-acting drugs) [30,31]. TMC278 corresponds to the recently approved NNRTI, rilpivirine [32]. Note that TMC181-LA is a prototype HIV long-acting protease inhibitor; however, the compound is not itself a candidate for clinical development. We tested TMC278-LA as adjunct to the backbone of 3TC and TDF for treating disseminated HIV infection and TMC278-LA alone or in combination with TMC181-LA for maintenance therapy in mice with suppressed HIV RNA. As expected, TMC278-LA was very potent in combination with the NRTI backbone (see above). However, TMC278-LA as monotherapy was not sufficient to maintain HIV suppression. Indeed, all mice showed a viral breakthrough. This prompt viral breakthrough is consistent with the clinical experience in humans: the NNRTI class must be given with a potent backbone [33]. Indeed, due to the long half-life of efavirenz (EFV) and nevirapine (NVP), as opposed to other anti-HIV drugs, interruption of ART containing either EFV or NVP at once results in a monotherapy with the risk of the emergence of NNRTI-resistant strains [34,35]. This clinical observation documents the relatively low genetic barrier of NNRTIs when given alone. In contrast, TMC278-LA in concert with TMC181-LA was highly efficient with a response rate close to 100% over time. In fact, clinical trials are under way to examine simplification of ART (e.g., combination of the protease inhibitor atazanavir and the integrase inhibitor raltegravir) [36]. Thus, this HIV mouse model would be superbly suited for a pre-clinical proof-of-concept of novel ART strategies (e.g., nucleoside sparing regimens, long-acting drugs).

Conclusions

In summary, we present data of ART responses/failures in larger number of mice that eventually position this hu mouse model as key tool in the evaluation of novel treatment strategies and latency. Hu mice, indeed, recapitulate central steps in HIV infection, including high-titer viral dissemination, response to ART, viral failure in the case of non-adherence and very importantly viral rebound after ART interruption. Viral rebound after interruption of ART points clearly to a latent reservoir of HIV. This model will be crucial when testing compounds for activating the latent reservoir aiming to eradicate and eventually cure HIV. We underscore this statement by documenting the value of long-acting anti-HIV drugs for suppressing HIV that might be very effective in certain clinical situations, e.g., PREP, PEP or in patients with poor adherence, as a simplified maintenance regimen, or in patients unable to swallow drugs.

Materials and Methods

Ethics statement

All experiments as well as procurement of human cord blood were approved by ethical committees of the University of Zurich and the Federal Veterinary Department and. The experiments were conducted according to local guidelines (TschV, Zurich) and the Swiss animal protection law (TschG). Human cord blood was collected with informed written consent of the parents.

Generation of hu mice

Immunodeficient NOD/shi-scid/$\gamma_c$null (NOG) [19] mice were reconstituted and infected as described [9]. Briefly, newborn NOG mice were irradiated 1–3 days after birth with 1 Gy and subsequently injected intra-hepatically with $2.5\pm0.5\times10^5$ CD34+ cells. CD34+ cells were isolated from human cord blood with immunomagnetic beads (Miltenyi Biotec) with an yield of $0.5–4\times10^6$ CD34+ cells from one donation (purity >90%). CD34+ cells and "non-target" fractions were stored frozen in liquid nitrogen until use. At around 15 weeks after transplantation, the engraftment of human immune cells was checked by staining peripheral blood mononuclear cells for the panhuman marker CD45. In all experiments, mice were randomized into mock- or ART treated groups.

Generation of food pellets containing anti-HIV drugs

Food pellets were made by mixing 2.5 g of 3TC, TDF and AZT each, and 5 g of RTV with 5 kg of ground protein-rich, vitamin-fortified food (Nafag 3432, Provimi Kliba AG, Switzerland) which

was subsequently formed to food pellets and sterilized by gamma-irradiation with 25 kGy. All batches of food pellets were analyzed for the correct amount of drugs admixed by HPLC (see below). Food and tap water were given ad libitum. TMC278-LA and TMC181-LA were generated by wet milling the compounds to get nanosizes and their subsequent formulation with non-ionic surfactants [30,31]. They were injected s.c. at 160 and 400 mg/kg, respectively.

HPLC-MS/MS method for measuring levels of TDF and 3TC in plasma and food pellets and TMC278 and TMC181 in plasma

Concentrations of drugs in the plasma and food pellets were determined by a qualified research liquid chromatography and mass spectroscopy (LC-MS/MS) method. For the analysis of diet, food pellets were diluted with water (1:10) and homogenized. Aliquots of each homogenate (50 µL) were solubilized with methanol (three volumes) and extracted with an identical volume of acetonitrile. Plasma samples (50 µL) were prepared identically as the food pellet homogenates. Plasma and food pellets were quantified using a specific LC-MS/MS method.

LC-MS/MS analysis was carried out on an API-4000 MS/MS (Applied Biosystems), which was coupled to an HPLC system (Agilent). The MS/MS was operated in the positive ion mode with the TurboIonSpray-interface (electrospray ionization) and optimized for the quantification of the compound (MRM transition for TDF: 520.2>270; for 3TC 288>176; for TMC278 367.2>224 and for TMC181 585.2>429).

The calibration range was flexible and depended on the study design. The limit of quantification was 0.5–10 ng/ml, depending on the compound. The accuracy (intra-batch accuracy for independent QC samples) was 80–120% of the nominal value over the entire concentration range of the samples.

HIV infection and ART

Mice were infected i.p. with HIV YU-2, 1×10^6 tissue-culture infectious dose$_{50}$ ($TCID_{50}$) per mouse. $TCID_{50}$ was determined in human CD8+T cell depleted PBMC from three donors which were stimulated by PHA and anti-CD3 beads (Dynal). HIV RNA plasma levels were measured by RT-PCR (AmpliPrep/COBAS TaqMan HIV-1 Test, Roche) at various times after infection.

Mice were monitored three times a week for symptoms or signs of adverse events, according to a standard score sheet.

Flow cytometry

Human cells, T cells and B cells were measured by flow cytometry of white blood cells stained for human CD45-APC, CD4-PerCP-Cy5.5, CD8-PB, and CD19-PE-Cy7 (all from BD Biosciences).

qPCR analysis of mouse organ samples

DNA and RNA from half of a spleen were extracted simultaneously with the AllPrep DNA/RNA Kit (Qiagen). DNA qPCR was as described [37], using HotStarTaq Master Mix (Qiagen), 1 µM of each primer and 0.1 µM FH probe. Experiments were done in duplicate with the real-time thermocycler IQ5 (BioRad) and as cycling profile: 95°C 15 min, 60× (95°C 5 s, 55°C 5 s, 60°C 40 s). The following oligonucleotides were used for HIV gag gene: mf319tq (probe): FAM5′-TGC AGC TTC CTC ATT GAT GGT-3′TAMRA [38], ts5′gag (sense): 5′-CAA GCA GCC ATG CAA ATG TTA AAA GA-3′ [37] and skcc (antisense) (5′-TAC TAG TAG TTC CTG CTA TGT CAC TTC C-3′ [39]. The following oligonucleotides were used for the reference gene GAPDH: mf70tq (probe): FAM5′-AAG GTC GGA GTC AAC GGA TTT GGT CGT-3′TAMRA, mf45 (sense) 5′-TCG ACA GTC AGC CGC ATC TT-3′ and mf46 (antisense) 5′-GGC AAC AAT ATC CAC TTT ACC AG-3′. Mean normalized expression (MNE) was calculated with qbase-PLUS (version 2.0, Biogazelle). The following oligonucleotides were used for HIV tat/rev gene mf84-YU-2 (sense) 5′-ACA GTC AGA CTC ATC AAA GTT CTC TAT CAA AGC A-3′ [37], mf226tq (probe): FAM5′-AGG GGA CCC GAC AGG CCC-3′TAMRA [37] and mf83 (antisense): 5′-GGA TCT GTC TCT GTC TCT CTC TCC ACC-3′ [37].

Since mice were infected with HIV-1 YU-2, the plasmid encoding YU-2 was used as standard in the PCR reactions with a detection limit down to 5 copies/reaction.

Reverse transcription

RNA was DNase treated using DNA-free kit (Ambion). For reverse transcription random hexamer primers (Operon Technologies) and SuperScript III reverse transcriptase (Invitrogen) were used. Reverse transcription was performed as described earlier [40]; briefly cDNA synthesis was performed using 10 µl DNase treated RNA in the presence of Ribolock Rnase inhibitor (Fermentas) in a total volume of 50 µl as follows: 60 min at 50°C, 60 min at 55°, 15 min at 70°C and then 1 min on ice. Subsequently 1 µl of RNAseH (NEB) was added to each tube and incubated at 37°C for 20 min. Aliquots were stored at −20°C or used immediately for real-time PCR analysis.

Ex vivo reactivation

Splenic cells of half of a spleen were thawed and split in two equal parts and then incubated in RPMI containing fetal calf serum (10%), IL-2 (10U/ml), penicillin (5%)/streptomycin (5%) and L-glutamine (5%) at 37°C for 12 hours. Subsequently cells were washed and then cultivated with or without mitogens (PHA at 3 µg/ml, PMA at 10 ng/ml), anti-CD3/CD28 beads (Dynabeads, Invitrogen) and IL-7 at 20 ng/ml. 18 hours later, DNA and RNA were extracted simultaneously with the AllPrep DNA/RNA Kit (Qiagen) which was then used for quantifying the HIV mRNA Gag transcripts.

Genotyping

Dideoxynucleotide-based sequence analysis was performed as described [41]. Briefly, Dideoxy sequencing reactions were performed on the purified amplicon (ABI Prism Big Dye Terminator Cycle Sequencing Kit, Version 3.1, Applied Biosystems) with a set of eight sequence-specific primers distributed over the PR-RT sequence for both strands: F1, 5′-GAGAGCTTCA GGTTTGGGG-3′; F2, 5′-AATTGGGCCTGAAAATCC-3′; F3, 5′-CCTCCATTCC TTTGGATGGG-3′; F5, 5′-CACTCTTTGG CAACGACCC-3′; R1, 5′-CTCCCACTCAG-GAATCC-3′; R3, 5′-CTTCCCAGAA GTCTTGAGTTC-3′; R5, 5′-GGGTCATAAT ACACTCCATG-3′; R6, 5′-GGAA-TATTGCTGGTGATCC-3′. Reactions were purified with a DyeEx Purification Protocol (Qiagen) and analysed with the ABI3730xl DNA Analyzer (Applied Biosystems). Sequence data files were grouped per sample identifier (ID) and aligned against the reference HXB2 reference sequence by means of the Sequencher TM Program V 4.1.4 (Gene Codes Corp.). A 25% mixture scoring rule (similar to 20% mixture identification by 454 deep sequencing) was used for the electropherogram analysis.

Calculations and statistics

Statistical analyses were performed using GraphPad Prism5.0 (GraphPad Software). Data were analysed by non-parametric Mann-Whitney test. In all figures, points represent values of individual mice, and lines depict mean values.

Supporting Information

Figure S1 Engraftment levels of hu mice before HIV infection. The mice were checked for engraftment levels at a median age of 132 days (25–75% percentiles: 103–136) as quantified by staining peripheral blood for the panhuman marker CD45. In addition, the percentage of CD4+, CD8+ and CD19+ cells were determined by flowcytometry.
(TIF)

Figure S2 PK data of AZT and RTV. (A and B) Plasma levels of AZT and RTV, respectively, over a day of mice on food pellets containing 0.5 mg/g or 1 mg/g food of AZT or RTV, respectively. The mice used for analysis of PK data have been on food pellets containing drugs for around 2 weeks for PK equilibration. The shaded area indicates the therapeutic range as defined in human. The different colours indicate the experiments done with the same food batch.
(TIF)

Figure S3 AZT at the dose applied was highly toxic. Mice were 2 weeks on a regimen with AZT as added at 0.5 mg/kg to the food pellets and were subsequently euthanized. Extensive laboratory chemistry and hematology work-up was done by the Institute of Clinical Chemistry and the Division of Hematology, USZ.
(TIF)

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Author Contributions

Conceived and designed the experiments: MN DB GK RS RFS. Performed the experiments: MN GGH AA DL MAR SB UH ES SR KA BS. Analyzed the data: MN BS GK DB RS RFS. Wrote the paper: MN RFS. Provided key reagents: KA AvC.

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3.2.2 High resolution mapping of HIV drug resistance evolution driven by APOBEC3G

Authorship contributions

I performed all the experiments and created figures 1 to 4. With Annette Audigé, Roberto Speck and Viviana Simon, I designed the experiments and wrote the manuscript. Viviana Simon performed the NGS and figures 5 and 6.

Introduction

Humans have evolved multi-layered defenses against invading pathogens. The APOBEC3 family of seven cytidine deaminase enzymes is part of the host's protective machinery [1]. HIV evolved, in turn, a number of strategies to evade precisely these antiviral mechanisms; in particular the HIV accessory protein Vif efficiently counteracts the antiviral activity of some of the most active APOBEC3 (e.g., A3G[1]).

However, proviruses with footprints of past deamination have been found in many HIV infected patients suggesting that Vif inactivation occurs frequently. Moreover, HIV strains with Vif alleles unable to completely neutralize APOBEC3 enzymes have been identified suggesting that complete neutralization is not necessary for survival of HIV as a population[2, 3]. Our collaborator Dr. Viviana Simon showed recently that HIV can exploit APOBEC3 to escape from nucleoside reverse transcriptase inhibitor (NRTI) lamivudine (3TC)[4]. For this, Dr. Simon and co-workers generated single nucleotide Vif-deactivating HIV mutants and found that partial A3G neutralization defects associated with Vif mutation 45G permit efficient replication *in vitro* in human peripheral blood mononuclear cells (PBMC). In the absence of any drug selection, these infections resulted, however, in a diverse proviral population in which 3TC drug resistance-associated mutations were found with high frequency[4]. These results suggest that hyper-mutated, defective proviruses can shape the phenotype of circulating viruses.

Reminiscent of observations in HIV infected patients[20], Vif-deficient HIV failed to spread in the humanized mice while proviruses derived from humanized mice infected with Vif-proficient HIV harbored numerous G-to-A mutations dinucleotide contexts preferred by

several distinct APOBEC3 molecules (GG or GA[21]). It has been a point of debate whether APOBEC3 driven mutagenesis contributes to the emergence of drug resistance[24].

To address the question of how much does APOBEC3 deamination contribute to overall heterogeneity of HIV genomes, we choose to use antiretroviral drugs to perturbate the *in vivo* infection upon dissemination. This experimental system permits to create an environment in which viral escape has an immediate and measurable fitness advantage (i.e. emergence of drug resistant viruses).

Material and methods

In this section I will only focus on the parts that are not covered in the rest of the thesis.

Cells and reagents

293T cells and TZM-bl cells were obtained from the NIH; they were cultured in DMEM medium (Lonza, Switzerland) supplemented with 10% FBS (Lonza, Switzerland), 1% penicillin (Life technologies) and 1% streptomycin (Life technologies). MT-4 cells were also obtained from the NIH and were cultured in RPMI medium (Lonza, Switzerland) supplemented with 10% FBS (Lonza, Switzerland), 1% penicillin (Life technologies) and 1% streptomycin (Life technologies).

3TC (Lamivudine) was purchased from GlaxoSmithKline (Epivir tablets). Tablets were grinded and weighed according to the 3TC content. 3TC was added to the food pellets as previously described[7]

Viral stocks

Viral stocks were generated by transfecting 293T cells with proviral DNA encoding the various Vif-mutants within the backbone of NL4-3 (obtained from the NIH) using PEI, and were washed 18 hours post-transfection. The 45G and the SLQ mutants were generated by site directed mutagenesis, using mutagenic primers and PCR techniques, replacing the codon 45 by GGA nucleotides and the 144 to 146 codons by GCTGCAGCG nucleotides, respectively, as previously described[2, 25, 26].

Viral supernatants were collected 48 hours post-transfection, filtered and frozen at -80°C until further use. Gag p24 concentration of viral stocks was quantified using an in-house ELISA[27] Tissue culture infectious dose 50 (TCID50) were determined by infection of TZM-bl with serial dilutions of viral stocks.

Amplification of HIV from plasma virions

RNA was extracted from 200ul plasma using Qiagen viral RNA kit as per manufacturer's instruction.

For the deep sequencing approach, cDNA was synthesized using the Thermoscript Kit (Invitrogen) and antisense ID cDNA primers (1528 5'-TTGCCYAATTCAATTTNNNNNNNNATTTCTGTATGTCATTGACAGTC, machine mixed; 1468 5'- TTGCCCAATTCAATTTNNNNNNNNNATTTCTGTATGTCATTGACAGTC, handmixed). The RT reaction was performed for 60 minutes at 55°C. cDNA products were column purified and eluted in 10ul H20.

For the amplification of complete Vif coding region, RNA from plasma collected at baseline or at later time points (week 8 or week 10) was reverse transcribed using random hexamers as described[29]. The full-length Vif region was amplified as previously described[29] and cloned using Strataclone blunt kit[30]. 6-12 individual clones were sequenced and manually aligned to Vif NL4-3 sequence.

MiSeq Illumina

The amplified PCR product containing the specific illumina adapters was run on a bio-analyzer for quality control and quantity determination. 37 to 47 samples were diluted to 10nM and further processed for MiSeq multiplexing sequencing (Illumina Inc., San Diego, CA). 60% of PhiX library (Illumina Inc., San Diego, CA) was spiked into the samples to add diversity. Runs generated 3.5 to 6 million paired-end reads (2 × 150 bp) covering the 47 HIV samples (37-47) as well as PhiX. Of the 13 million reads, over 6 million reads matched the HIV samples with a minimum of 13,017 reads per sample.

The processing of the sequence data was done using several custom scripts (a mix of shell and perl), developed specifically for this project. The sequences were binned into separate barcodes. For each set, the PCR-primers and tags were identified in the sequenced reads, and

each unique combination of HIV sequence and tags were identified and assigned a count, which was the frequency of occurrence of the sequence-tag combination in the sequencing data. This frequency represents the clonal amplification of the molecule and the multiple occurrences are just counted once. After this compression of the data, the mutations in the sequences are identified and accurate inventory of the various mutations in each sample is made, and this allows us to identify the dynamics of the mutations under different treatments.

Results and Discussion

Partial neutralization of A3G permits replication in cell culture

In order to verify that the mutant viruses are able to replicate in the presence or absence of APOBEC3 proteins *in vitro*, we infected MT4 T cells as well as mitogen activated (PHA 1µg/mL for 48 hours) human CD34 negative cord blood mononuclear lymphocytes (CBL) with HIV WT, HIV Vif 45G and SLQ isogenic Vif mutant viruses. Of note, HIV WT is able to counteract A3G and A3F, HIV 45G is not able to counteract A3G and SLQ is not able to counteract A3G nor A3F and therefore has been considered as an equivalent of a Vif-deficient virus[26, 31, 32] (Fig. 1A). The three viruses replicated to similar levels in MT4 cells, which do not express any A3G[31]: 45G and SLQ replicated to a similar level as WT in the absence of A3G (MT4 cell line) but only WT and 45G replicated efficiently in primary cells, which naturally express A3G. In agreement with previous reports, HIV SLQ, which cannot counteract any of the APOBEC3, is highly attenuated in the presence of APOBEC3 (Fig. 1B).

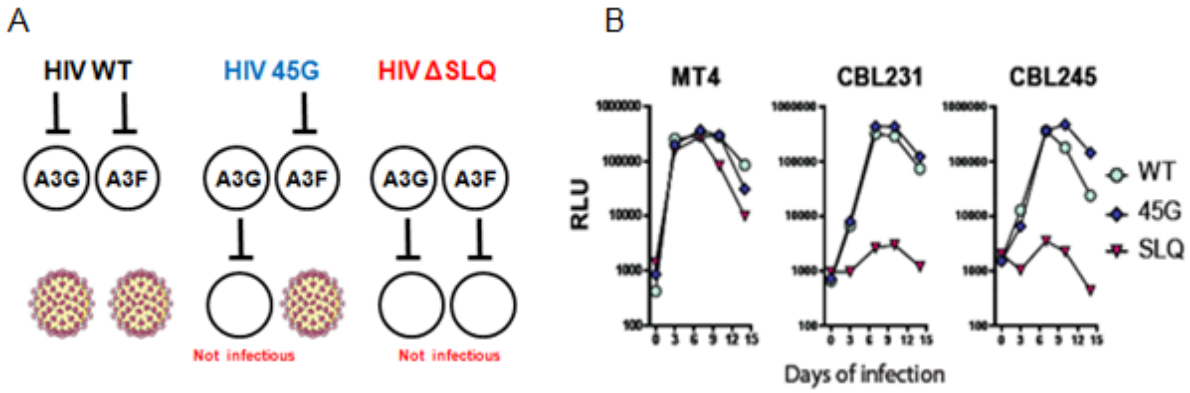

Figure 1: HIV Vif mutant viruses engineered to display selective APOBEC3 neutralization defects. A. Model of differential APOBEC neutralization of HIV WT, HIV-

45G and HIV-SLQ. B. Spreading infection of the three viruses in primary cord blood lymphocytes.

Partial neutralization of A3G is associated with lower viral fitness and complete lack of APOBEC3-neutralization leads to a lack of viral replication *in vivo*

To assess the impact of differential neutralization of APOBEC3G/F on HIV infection *in vivo*, we infected humanized mice with the three different viruses and monitored viral replication weekly over four weeks by measuring plasma viral load and determined the peripheral blood CD4/CD8 T cell ratio overtime. As observed *in vitro*, no replication was detected for the SLQ mutant (Fig. 2A), which is in line with recent work showing lack of replication in humanized mice infected with Vif-deficient CCR5-tropic HIV strains[21, 33]. Therefore, this experiment proves convincingly that Vif without its SLQ box which binds to the proteasomal machinery has no anti-APOBEC3 activity, and behaves indeed like a Vif-deficient virus. In contrast, the 45G mutant showed a replication pattern *in vivo* that was different from the one observed *in vitro*: viral replication was similar to the one of the WT virus at 4 weeks, but significantly lower thereafter (P=0.014 and P=0.0039 respectively), indicating a progressive decrease in viral fitness overtime. However, the pathogenicity of the 45G mutant was as high as the one of the WT virus, as seen by the similar ratio of CD4/CD8 T cells in peripheral blood overtime, thus, factors other than solely HIV viremia contributed to the progressive CD4+ T cell loss (Fig. 2B). The difference in the viral fitness of 45G between the *in vitro* and *in vivo* experiments is likely explained that 45G's attenuation takes longer to become apparent, corroborating the value of studying HIV pathogenesis in humanized mice. The attenuation observed is most likely explained by a slow accumulation of deleterious mutations overtime in several proteins of the 45G virus due to the unopposed editing effect of A3G [34, 35]. We cannot exclude an additional non-editing effect of A3G in this partial inhibition, like RNA binding and subsequent inhibition of cDNA accumulation and integration in the host's genome, as described previously *in vitro*[36-38]. Notably, the catalytic domain of A3G, responsible for its cytidine deaminase activity and the region responsible for the non-editing effect are at different locations[36, 39, 40].

A B

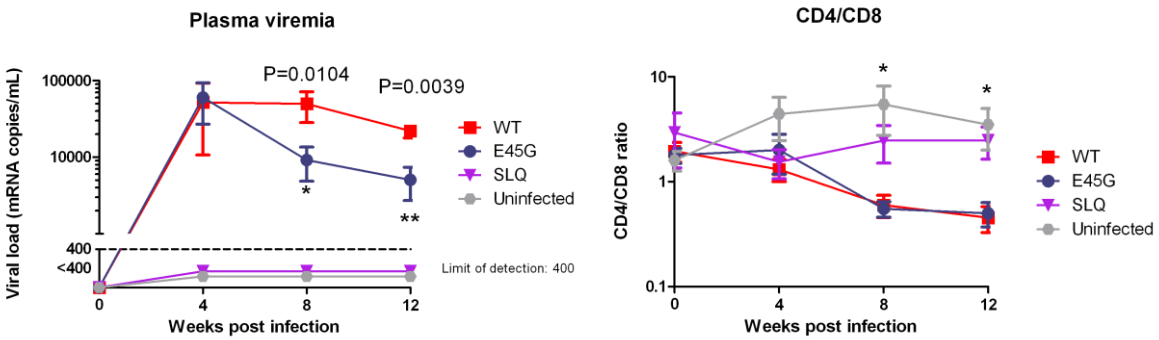

Figure 2. Partial neutralization of A3G is associated with lower viral fitness and complete lack of APOBEC3-neutralization leads to a lack of viral replication *in vivo*. A. Viral replication of Vif mutants in humanized mice. Immune reconstituted mice were infected with either NL4-3 WT (n=10), NL4-3 45G (n=9), NL4-3 SLQ (n=4) or not infected (n=4) 12 weeks post reconstitution. Plasma viremia was measured over time. Dots represent mean of the plasma viral loads for each group at each time point and bars represent SEM. B. CD4/CD8 ratio over time. The ration of the frequencies of CD4+ and CD8+ T cells in peripheral blood of the infected mice was determined by immunostaining and flow cytometry weekly over 12 weeks post infection. Dots represent mean of the ratios for each group at each time point and bars represent SEM.

Partial neutralization of A3G *in vivo* has no cost on viral fitness under selective pressure

To better understand how partially active HIV Vif alleles can exploit APOBEC3 to escape from particular antiretrovirals as shown previously *in vitro*[4] and in HIV-infected individuals failing antiretroviral treatment[3], we treated HIV-infected humanized mice with 3TC for six weeks starting four weeks post infection and monitored viremia and peripheral blood CD4/CD8 T cell ratio every two weeks during the treatment period (Fig. 3A). Two weeks after the start of the treatment, viremia was decreased in all WT-infected mice and in 5 of 6 45G-infected mice (Fig. 3B). The mouse #120 that showed no decrease on the viral load had a higher viral load as compared to the other animals. It showed signs of wasting and

unfortunately it had to be euthanized. Between week 6 and 10 post infection, virus rebounded, in particular in the 45G-infected group.

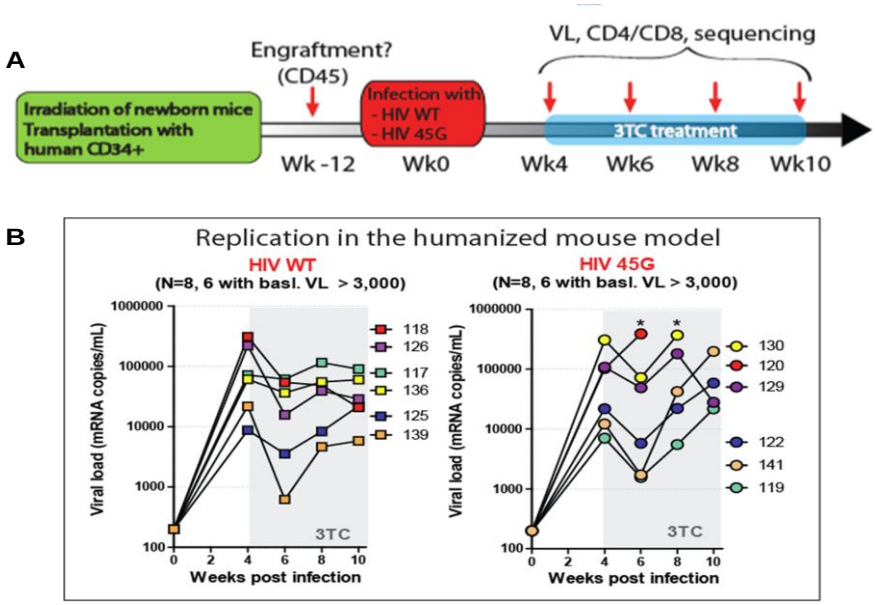

Figure 3: The humanized mouse model provides an *in vivo* model for HIV replication. A. The experimental time line is depicted. Eight immune reconstituted mice were infected with either NL4-3 WT or NL4-3 45G, 12 weeks post reconstitution. 3TC was added to the food pellets starting week 4. Plasma viremia and CD4+ T cells were measured over time. B. Replication of HIV WT and HIV 45G in humanized mice. Viremia in the plasma was measured over a 10 week period. The left panel summarizes the replication of WT viruses and the right panel the replication of the 45G viruses. Productive infection (>3,000 copies/ml) was observed in six out of eight animals in each group.

A comparison of the difference in viral load between either week 4 and 6 or week 6 and 8 between HIV WT- and 45G-infected mice revealed that the decrease of viral load from week 4 to 6 was similar in both groups (Fig. 4A and 4B, left panel). Thus, the Vif mutation did not affect the initial response to 3TC. In contrast, viral rebound between week 6 and 8 was significantly stronger for the 45G group, which is consistent with more rapid emergence of 3TC resistance. One would spontaneously assume that the unopposed editing effect of A3G resulted in a more rapid and higher diversification of HIV, and in particular at the position 184, rendering the 45G mutant more apt to replicate in the presence of 3TC[4].

The peripheral blood CD4/CD8 T cell shows the same pattern for both groups: the ratio remained constant until the start of the 3TC treatment, increased between week 4 to 6 (when viremia decreased for both viruses (Fig. 3B)), and decreased thereafter (when viremia increased again (Fig. 4C)). Thus, also under selective pressure the 45G is as pathogenic as the WT virus.

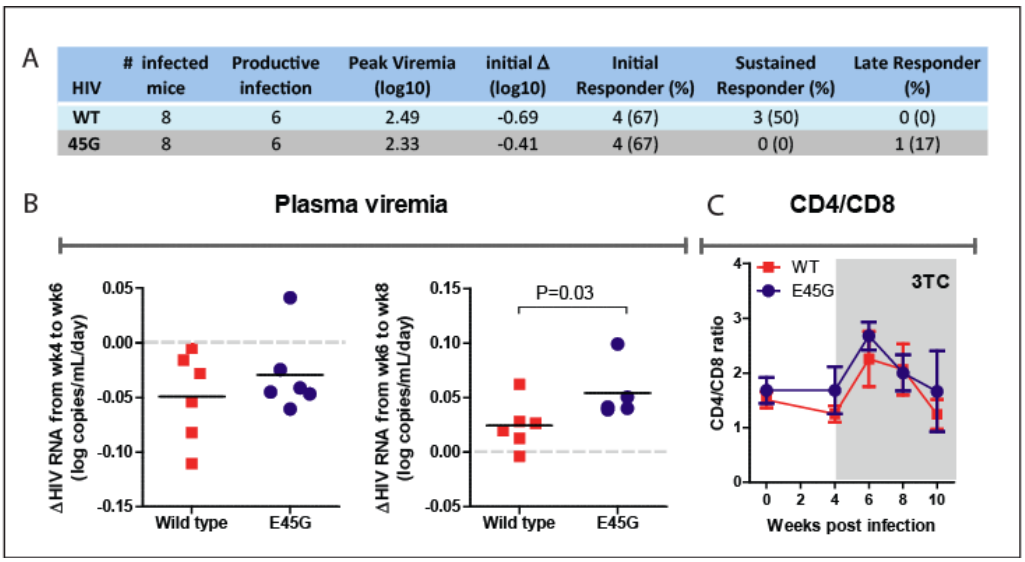

Figure 4: Overview of the virological and immunological characteristics of HIV WT and HIV 45G infection in humanized mice. A. Summary of virological parameters of infection and lamivudine treatment responses. B. Plasma viral load changes depicted. Dots represent individual mice and lines represent means. The grey line identifies no change. C. CD4/CD8 ratio over time. Dots represent mean of infected mice for each group at each time point and bars represent SEM.

Kinetics of viral drug escape in mice with A3G resistant and A3G sensitive viruses.

In order to determine if resistance to 3TC appeared on 45G viruses faster than WT viruses, the RT region containing the codon 184 was sequenced, since mutations in this codon are responsible for 3TC resistance. While the WT virus codes for Met in position 184 (ATG), mutations induced by RT or A3G correspond to Ile (ATA) and mutations induced solely by RT correspond to Val (GTG or GTA). Amazingly, the input HIV strain was replaced already after 2 weeks of 3TC treatment to a great part by viruses with resistance signature of 3TC and this irrespective of the HIV strain used to challenge the mice (Fig. 5). This rapid emergence of

the M184I/M184V resistance mutations is rather identical to the resistance emergence in HIV-infected patients under 3TC monotherapy[41].

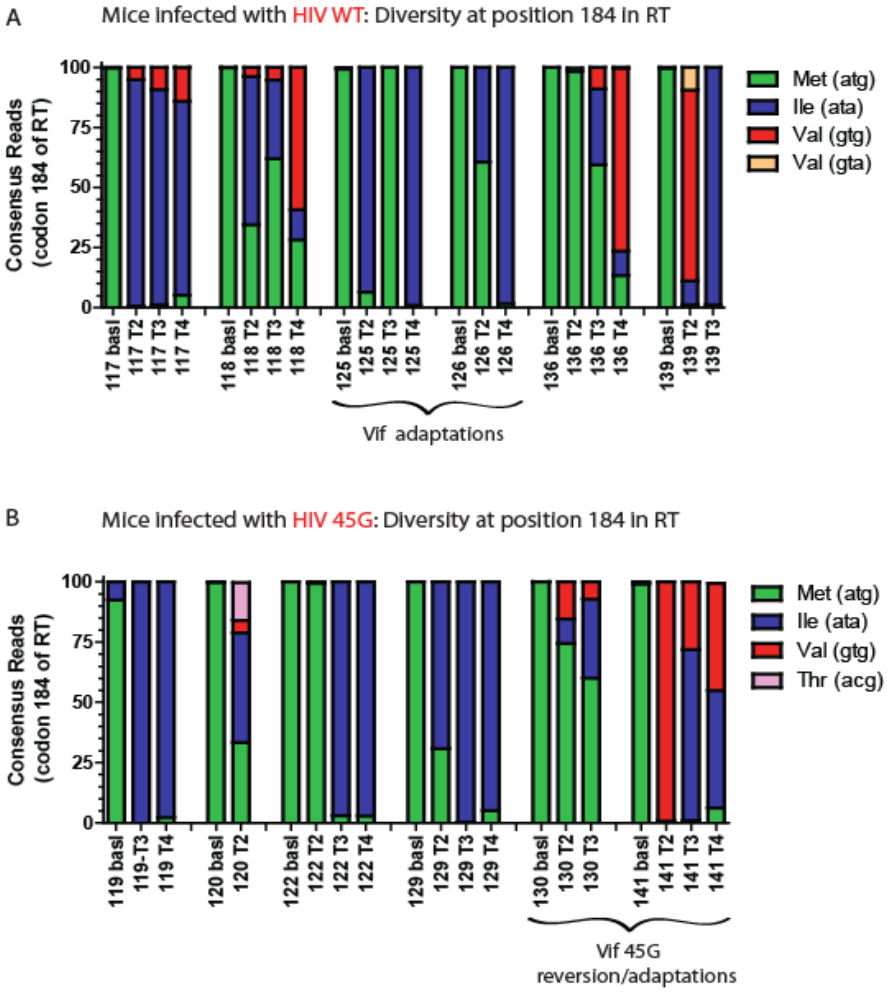

Figure 5: Emergence and evolution of 3TC resistance in the plasma of mice infected with HIV WT or HIV 45G. A. The frequency of mutations at codon 184 of RT is depicted for the mice infected with WT virus. B. The frequency of mutations at codon 184 of RT is depicted for the mice infected with 45G virus. Red virus results from RT errors whereas blue virus is the result of RT as well as A3G. Green virus is WT.

Looking closer at the type of resistance, we noted that a mixed population of HIV strains emerged with M184I and M184V substitutions in mice infected with WT HIV and a rather homogenous population with mainly M184I substitutions in mice infected with the 45G mutant. HIV strains with M184V mutations emerged in mice infected with 45G only when

Vif reverted to WT (Fig. 6). Thus, it seems that the A3G editing process is a determining factor in the 45G mutant.

These results show that a partial neutralization of A3G (45G virus) does not induce faster drug resistance than WT virus *in vivo*. The Vif mutant 45G has been found in HIV-infected patients. *In vitro* analyses showed that it can partially neutralize A3G [2]. Additionally, this mutant was able to develop the 3TC resistance signature mutation, prior to 3TC exposure and more interestingly, it was able to transfer this resistance to the WT populations and shape populations via recombination, *in vitro* [4]. However, this experiment was performed in a very sophisticated way, by infecting a cell line with both viruses WT and 45G and then the cells were treated with a high dose of 3TC [4]. In this way, viral replication and recombination took place. Proof was lacking that these pathogenic events to happen *in vivo*.

The mutation M184I increases the RT fidelity 4-fold and M184V 2.5-fold as compared to WT RT[42, 43] and thus viruses with M184I or M184V diversify less than WT HIV. The higher fidelity of the RT with M184I may prevent an excessive RT-driven diversification and thus, HIV strains with M184I might have a replication advantage as compared to HIV strains with M184V or WT RT. The gain of RT fidelity may outweigh the A3G editing effects in those mice infected with HIV strains deficient in the neutralization of A3G. However, the gain of viral fitness of the 45G mutant is only apparent under drug or immune pressure promoting specific mutation as illustrated here with 3TC.

In two previous studies looking at the impact of different A3 proteins on HIV mutagenesis, A3-induced mutations were detected only in proviral DNA, consisting largely of stop codons, but in plasma HIV RNA no signs of hypermutation were found and no signs of drug resistance mutations were present[21, 33]. The major difference, again, is that we induced a selective pressure by treating the mice with 3TC that uncovered the significance of A3G-driven mutations for HIV's adaptation to drug or immune pressure. We have to be aware that the immune response in humanized mice is only partially effective; in particular the cytotoxic T lymphocytes (CTL) activity is impaired [44], Thus, the HIV RNA mutations we detected are induced merely due to the drug pressure – the concept we show here will certainly be valid also for the well known phenomenon of immune escape in humans.

Regions in Vif that adapt and evolve in order to counteract A3G

In order to determine if the Vif gene was mutated or not through the experiment where we treated the mice with 3TC, we decided to sequence the Vif gene at baseline and at late points of infection (weeks 8 or 10 post infection). The baseline sequences (before 3TC treatment) showed no evidence of mutation for both WT and 45G mutant. We found evidence for Vif reversion/adaptation in two mice infected with 45G and in two mice infected with WT (Fig. 6). For the mouse #130 (45G mutant), we observed mutations at positions G45E (reversion), from GGA to GAA (possibly A3G mediated), V10M from GTG to ATG (possibly RT mediated) and R90K from AGG to AAG (possibly A3F mediated). For mouse #141 (45G mutant) we observed a mutation R41K from AGA to AGG (possibly RT mediated). For the mouse #126 (WT virus), the mutation was N190K from AAT to AAG (possibly RT mediated) and for mouse #125 (WT virus). Interestingly, both pairs of mice stand out with respect to the pattern of drug resistance (Fig. 5) and all of these mice, except #141, were reconstituted with the same cord blood.

Intriguingly, the 45G mutant viruses showed APOBEC3-driven as well as stochastic mutations, as compared to the WT, which only exhibited stochastic mutations, pointing to the extremely fine-tuned editing machinery of the APOBEC3 system. The Vif gene, itself, is located 3' of the central polypurine tract (cPPT) and excels by a very low A3G-driven mutational rate as compared to other HIV genome regions; this is most likely due to the fact that this region is only present for a very short time as single stranded DNA (ssDNA) during reverse transcription rendering it a short-lived target of A3G[45]; notably, A3G acts solely on ssDNA [46-48]. Thus, the APOBEC3-driven mutations we observed speak in favor that they were induced by the strong selective pressure we applied by putting the mice on 3TC. On the other hand, the RT is located 5' of the cPPT, which corresponds to the hot spot of A3G-activity, since this region is present as ssDNA for longer time [45] and explains the higher amount of mutations found in the RT region

Taken together, these results show that under selective pressure (3TC treatment, in this case) Vif is able to adapt, in particular the 45G mutant with A3G/A3F and RT-mediated mutations and the WT with only RT-mediated mutations.

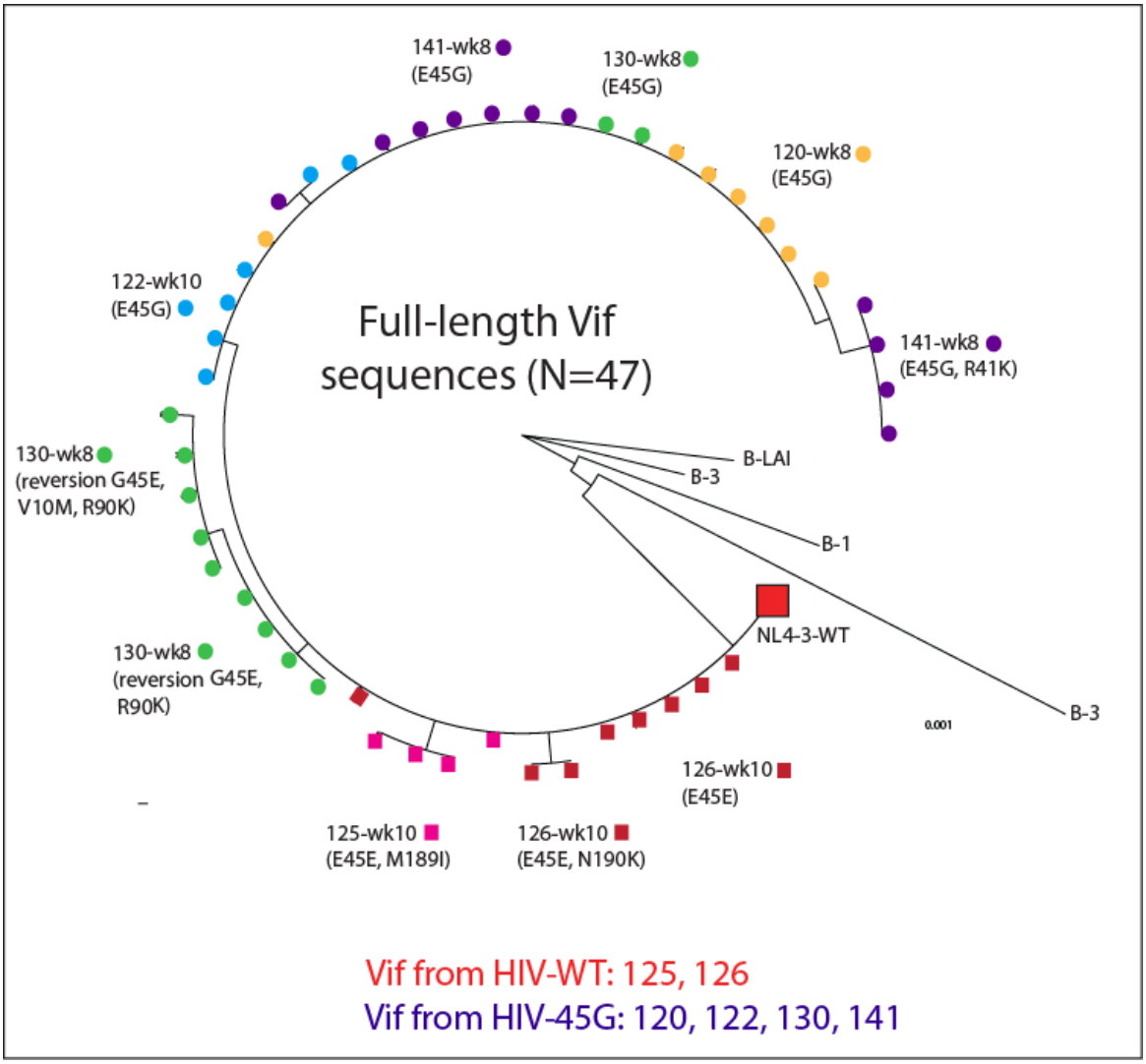

Figure 6. Evidence for adaptations of HIV Vif. A total of 47 individual sequences were performed in the full-length Vif gene for mice infected with HIV-WT: 125 and 126 and for mice infected with HIV-45G: 120, 122, 130 and 141. These sequences were aligned and compared to the NL4-3 WT used at baseline to infect mice 125 and 126 using a phylogenetic tree. Codon 45 of the RT is depicted for each sequencing performed. For 45G viruses it corresponds to E45G, for WT viruses it corresponds to E45E and for mutant viruses undergoing a reversion it corresponds to G45E. Other mutations observed in the Vif region are also indicated. Each dot represents an individual sequence, each color represents a different mouse

Concluding remarks

This work also illustrates very convincingly the power of the Illumina platform for next generation sequencing HIV quasi-species from the plasma and in particular for detecting resistance mutations. Before 2007, the former sequencing studies in the field of HIV used rather exclusively the Sanger's sequencing-based PCR amplification, which permitted to identify the sequence of the most prevalent HIV strain but did not allow to discriminate quasi-species or to detect minor HIV populations. In addition, samples were subjected to *Taq* polymerase amplification errors [49, 50]. In the humanized mouse work described above by Sato et al. and Krisko et al. [21, 33], conventional Sanger's sequencing-based PCR amplification was used. This could explain the striking difference between the papers by Sato et al. and Krisko et al.: Sato et al. found A3-driven mutations in viral DNA from splenocytes when mice were infected with WT virus [21] while Krisko et al. found only such mutations in viral DNA for Vif-deficient virus from PBMCs, spleen, liver and lung [33].

Nowadays, the 454 next generation sequencing is widely used to detect resistance mutations [51-54]. In direct comparison, the next generation sequencing techniques 454, PacBio RS (Pacific Biosciences), Illumina, and Ion Torrent (Life Technologies) were similarly efficacious for determining HIV co-receptor tropism [55]. In another work, two next generation sequencing techniques were compared: 454 from Roche and Illumina, using a mixture of ten HIV clones [56]. The authors concluded that for fixed costs, Illumina reads can provide a higher coverage and allow for detecting variants at lower frequencies.

Other experiments also showed that the Illumina platform offers a better coverage of the viral genome compared to other methods, providing an improved characterization of the heterogeneity present in the studied samples [57-59]. For instance, it provides up to 3 billion reads per run, as compared to 1 million for the 454 sequencing and the cost per 1 million bases can be up to 100 times cheaper than the 454 sequencing [60, 61]. For HIV infection, it is very important to cover all or the big majority of the sequences present, due to the quasi-species populations present on each patient, creating a very heterogeneous viral population.

In summary, our results show that the lack of counteracting A3G leads to mutations in the HIV genome in several genes, which is primarily apparent under selective pressure, as exerted here by 3TC treatment. The selective pressure presented in this work could also apply to the

CTL immune pressure in HIV-infected patients. These mutations generated might be then transferred to the circulating populations and thereafter shape the viral population overall, as shown previously *in vitro*[4], although this remains to be determined *in vivo.*

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3.2.3 Gene Therapy against HIV-1 using the humanized mice

ART is the standard treatment against HIV-1, which does not cure HIV-1 and has the disadvantage of side events, emergence of drug-resistant strains, a need for life-long intake, psychological dependence, and cost. Gene therapy is among the most promising strategies to cure HIV-1. In this work we tested the gene therapy in the hematopoietic precursor cells of human origin (CD34+) that are transplanted into the humanized mice in order to generate an immune system of human origin. Since HIV-1 requires the co-receptor CCR5 for viral entry, we engineered a lentiviral vector containing miRNA against CCR5, we transduced the CD34+ cells with this vector and evaluated the protection of the gene therapy against HIV-1. The results of this work have been recently published into the Journal of Virology and are found below.

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Own contribution:

I assisted Renier Myburgh for all experiments *in vivo* (humanized mouse), including the isolation of CD34+ cells from cord blood, the transplantation of the newborn mice with CD34+ cells, bleeding of the mice, periodic health check of the mice, euthanasia of the mice and processing of the organs.

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Lentivector Knockdown of CCR5 in Hematopoietic Stem and Progenitor Cells Confers Functional and Persistent HIV-1 Resistance in Humanized Mice

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ABSTRACT

Gene-engineered CD34$^+$ hematopoietic stem and progenitor cells (HSPCs) can be used to generate an HIV-1-resistant immune system. However, a certain threshold of transduced HSPCs might be required for transplantation into mice for creating an HIV-resistant immune system. In this study, we combined CCR5 knockdown by a highly efficient microRNA (miRNA) lentivector with pretransplantation selection of transduced HSPCs to obtain a rather pure population of gene engineered CD34$^+$ cells. Low-level transduction of HSPCs and subsequent sorting by flow cytometry yielded >70% transduced cells. Mice transplanted with these cells showed functional and persistent resistance to a CCR5-tropic HIV strain: viral load was significantly decreased over months, and human CD4$^+$ T cells were preserved. In one mouse, viral mutations, resulting presumably in a CXCR4-tropic strain, overcame HIV resistance. Our results suggest that HSPC-based CCR5 knockdown may lead to efficient control of HIV *in vivo*. We overcame a major limitation of previous HIV gene therapy in humanized mice in which only a proportion of the cells in chimeric mice *in vivo* are anti-HIV engineered. Our strategy underlines the promising future of gene engineering HIV-resistant CD34$^+$ cells that produce a constant supply of HIV-resistant progeny.

IMPORTANCE

Major issues in experimental long-term *in vivo* HIV gene therapy have been (i) low efficacy of cell transduction at the time of transplantation and (ii) transduction resulting in multiple copies of heterologous DNA in target cells. In this study, we demonstrated the efficacy of a transplantation approach with a selection step for transduced cells that allows transplantation of an enriched population of HSPCs expressing a single (low) copy of a CCR5 miRNA. Efficient maintenance of CD4$^+$ T cells and a low viral titer resulted only when at least 70% of the HIV target cells were genetically modified. These findings imply that clinical protocols of HIV gene therapy require a selective enrichment of genetically targeted cells because positive selection of modified cells is likely to be insufficient below this threshold. This selection approach may be beneficial not only for HIV patients but also for other patients requiring transplantation of genetically modified cells.

Combined antiretroviral therapy (cART) changed the face of HIV medicine: patients have a life expectancy close to that of uninfected people (1). However, cART has major disadvantages, including adverse events, emergence of drug-resistant strains in patients with poor adherence, a need for lifelong intake, psychological dependence, and cost. Thus, cART has not halted the pandemic (http://www.who.int/hiv/en/), and alternative therapies are needed to cure HIV.

Gene therapy has been widely discussed as a possible strategy to cure HIV and has been tested in phase I and II clinical trials. Autologous CD4$^+$ T cells (2, 3) or CD34$^+$ cells (4, 5) were gene engineered to express various anti-HIV moieties, including a combination of three RNA-based anti-HIV moieties (tat/rev short hairpin RNA [shRNA], TAR decoy, and CCR5 ribozyme) (4), a tat-vpr-specific anti-HIV ribozyme (5), and a conditionally replicating lentiviral vector expressing a long antisense to HIV (3), or were gene edited by zinc finger nucleases for CCR5 knockout (2). Gene engineering also generated HIV-specific CD4$^+$ or CD8$^+$ T cells (6, 7). Overall, the effects on HIV infection were modest, but importantly, gene engineering proved to be safe in humans.

The concept of engineering an HIV-resistant immune system received new impetus from the "Berlin patient," who was infected with HIV and was treated with hematopoietic stem cell transplantation for acute myeloid leukemia. He received bone marrow from a donor homozygous for the Δ32 CCR5 mutation, and thus, the progeny cells did not express CCR5. His case was the first in which a cure for HIV was documented (8) and provided hope that elim-

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inating CCR5 from the cell surface would be the "Holy Grail" for the cure of HIV. However, another HIV-infected patient suffering from anaplastic large-cell lymphoma also received a stem cell transplant from a homozygous CCR5-null donor. Unfortunately, in that case, X4-tropic HIV strains emerged that necessitated the reinitiation of cART (9).

In view of the modest success of phase I and II clinical trials and the data from stem cell transplantation, preclinical studies are needed to define the best anti-HIV moieties and the minimal number of gene-engineered cells required to advance gene therapy in HIV. Humanized (hu) mice, which are generated by the transplantation of $CD34^+$ cells, are of particular value in this context. These mice excel in their multilineage hematopoiesis (10), are highly permissive to HIV (11), and allow for the gene engineering of human $CD34^+$ cells before transplantation (12). Indeed, various anti-HIV moieties have been investigated in hu mice as gene therapy options, including cellular factors, boosting the anti-HIV immune response, and the HIV genome itself (12). These mice were used *in extenso* to investigate the effects of targeting CCR5 by shRNA (13–15) or zinc finger nucleases (ZNF) (16). All these studies reported a decrease in CCR5 expression in circulating and tissue leukocytes, which were not permissive to HIV *ex vivo*, but only the study by Holt et al. reported a significant decrease of HIV RNA copy number *in vivo* (16). The other studies either did not analyze the effects on HIV infection *in vivo* (14) or demonstrated no effect on viral load (15). The results of Holt et al. revealed disruption of CCR5 in only ~20% of all $CD4^+$ T cells. The follow-up in that study was only 8 weeks. Gene engineering of hematopoietic stem and progenitor cells (HSPCs) with a lentiviral vector encoding the broadly neutralizing anti-HIV human antibody 2G12 showed suppression of HIV RNA but was studied only 7 days after a challenge with a virus containing the corresponding epitope (17); gene engineering of an HIV-specific T-cell receptor also lowered the HIV RNA but only modestly (18). A very elegant study with $CD34^+$ cells edited with an HIV-1 long terminal repeat (LTR)-specific Tre recombinase showed a potent lowering of HIV RNA activity after HIV challenge in hu mice (19). All these data are promising; however, we lack a long-term follow-up of the effects of anti-HIV gene therapy in hu mice, the number of gene-engineered $CD34^+$ cells needed in the various studies to obtain an HIV RNA lowering effect, and a detailed characterization of the hematopoietic system. A major advance was recently presented by Barclay et al., who purified gene-engineered $CD34^+$ cells via the expression of a truncated version of CD25 (20).

Various means are available for gene engineering of $CD34^+$ cells; each has its pros and cons. A great deal of experience exists with shRNA (21); potential cons may be its potential to trigger the innate immune system (22) and its less-than-absolute downregulation of the target gene. Targeted gene disruption by ZFN, Talen, or Crispr/cas has the advantage of complete disruption of the gene of interest (23–25); however, the modest rate of gene engineering of $CD34^+$ cells (26), the potential of off-target effects (27–29), and the lack of clinical experience represent substantial hurdles for wider use *in vivo*.

We recently reported a novel microRNA (miRNA)-based gene knockdown (KD) strategy with improved knockdown relative to that obtained by methods conventionally used (30). A triple-hairpin cassette targeting CCR5 resulted in >90% CCR5 knockdown upon single-copy transduction in HeLa cells. The aim of the present study was to assess whether gene engineering of $CD34^+$ cells with this vector construct results in downregulation of CCR5 in progeny cells in hu mice and whether it could protect against HIV challenge *ex vivo* as well as *in vivo*. Since there is some evidence in the literature that the number of gene-engineered $CD34^+$ cells is a major determinant of the success of the anti-HIV moieties (15, 16), we also made a major effort to generate hu mice with a very high number of gene-engineered $CD34^+$ cells. Finally, we characterized *in extenso* the hematopoietic system subsequent to HIV infection in hu mice with gene-engineered $CD34^+$ cells.

MATERIALS AND METHODS

Ethics statement. The procurement and use of $CD34^+$ cells from human cord blood were approved by the Cantonal Ethical Committee of Zurich (EK-1103). All adult subjects provided written informed consent. Animal care and experimental protocols were in accordance with the "Swiss Ethical Principles and Guidelines for Experiments on Animals" (http://www.akademien-schweiz.ch/en/index/Portrait/Kommissionen-AG/Kommission-fuer-Tierversuchsethik.html) and approved by the Veterinary Office of the Canton of Zurich, permit 26/2011. Manipulations of mice were in accordance with the regulations of the Veterinary Office of the Canton of Zurich (http://www.veta.zh.ch/internet/gesundheitsdirektion/veta/de/home.html).

Lentiviral vector production and titration. Lentiviral vector stocks were generated using transient transfection of HEK 293T/17 cells with the self-inactivating vector pCLX-R4-DEST-R2 encoding the microRNA to CCR5 (30), the psPAX2 plasmid encoding gag/pol, and the pCAG-VSVG envelope plasmid, as described previously (31). Lentivector titration was performed using transduction of HeLa cells, followed by quantification of green fluorescent protein (GFP)-positive cells 5 days after infection by flow cytometry as described previously (31).

Generation of humanized mice. NOD.scid.IL2R$^{-/-}$ (NSG) mice were bred and maintained in individual ventilated cages and were fed autoclaved food and water. Mice with a human immune system (humanized [hu] mice) were generated as described previously (32). Briefly, newborn (<5 days old) NSG mice received sublethal (1 Gy) total-body irradiation with a Cs source and then received 2×10^5 transduced or untransduced $CD34^+$ human HSPCs with a 50-μl Hamilton syringe via the intrahepatic (i.h.) route. For the fluorescence-activated cell sorter (FACS)-sorted R5 knockdown animals, $CD34^+$ cells were sorted postransduction into GFP-positive and -negative fractions, and then 2×10^5 $CD34^+$ GFP-positive or GFP-negative cells were injected i.h. into respective cohorts. All manipulations of hu mice were performed under laminar flow. Cell suspensions of the hu mouse organs were prepared in RPMI 1640 medium supplemented with 2% fetal calf serum.

HIV stock and infection of mice. Viral stocks were obtained by polyethylenimine (PEI)-mediated transfection (Polysciences) of 293T cells with either pYU-2 (R5 tropic) or JRCSF (R5 tropic) (provided through the NIH AIDS Research and Reference Reagent Program). At 48 h after transfection, virus was harvested, filtered (0.45 μm), and frozen at −80°C until use. Viral titers were determined as described previously (33). Briefly, 50% tissue culture infectious dose ($TCID_{50}$) was determined by infecting human $CD8^+$ T-cell-depleted peripheral blood mononuclear cells (PBMCs) from three donors that were stimulated by adding interleukin-2 (IL-2), phytohemagglutinin (PHA), and anti-CD3 beads (Dynal 11131D; Life Technologies). Mice were infected intraperitoneally (i.p.) with either HIV YU-2 or JRCSF at 2×10^5 $TCID_{50}$s per mouse. Plasma HIV RNA levels were measured by reverse transcription-PCR (RT-PCR) (AmpliPrep/COBAS TaqMan HIV-1 test; Roche) at various times after infection.

Flow cytometry. The cells in whole blood were counted in a Beckman cell counter. Cell suspensions were labeled with anti-human monoclonal antibodies (MAb) targeting the following cell surface markers: CD45-peridinin chlorophyll protein (PerCP), CD3-allophycocyanin (APC), CD4-Pe Cy7, CD8-BVa, CCR5-phycoerythrin (PE), CD34-APC, CD45RA-APC, and CCR7-PE (all from BD Biosciences or

Biolegend). Washing and reagent dilutions were done with FACS buffer (phosphate-buffered saline [PBS] containing 2% fetal calf serum and 0.05% sodium azide). All acquisitions were performed on a Cyan ADP (Beckman Coulter) flow cytometer. Data were analyzed with FlowJo software (Ashland, OR). Cellular debris and dead cells were excluded by their light-scattering characteristics. Transduced $CD34^+$ cells were sorted according to intrinsic GFP expression as measured by a BD FACSARIA III cell sorter.

HIV challenge *ex vivo*. Spleens of five hu mice transplanted with HSPCs gene engineered with the microRNA to CCR5 were dissociated through a nylon mesh, and red blood cells were lysed with ACK buffer (Lonza) for 3 min. Cells were sorted (≥99% pure) with an ARIA sorter (BD Bioscience) into GFP-positive and -negative cells and were subsequently activated for 24 h with PHA (4 mg/ml) in RPMI 1640 culture medium supplemented with IL-2 (100 U/ml) and 10% fetal calf serum. Thereafter, cells were infected with a $TCID_{50}$ of 3.3×10^5/ml of YU-2 for 6 h and washed three times. The supernatant of the last wash was used as the baseline p24 antigen level measured by an in-house enzyme-linked immunosorbent assay (ELISA) (34). Virus spread was then monitored at days 1, 4, 6, 8, and 10 postinfection.

Analysis of HIV envelope sequences. (i) Nucleic acid extraction. For viral envelope sequencing, total nucleic acid was extracted from 60 μl of mouse plasma on an EasyMag extractor (bioMérieux, Switzerland) according to the manufacturer's instructions. The elution volume was 50 μl.

(ii) Reverse transcription and PCR. For cDNA synthesis, 9 μl of extracted nucleic acid was reverse transcribed in a total reaction volume of 20 μl at 42°C for 30 min using a sequence-specific primer, MSR5 (35), and the PrimeScript One Step RT-PCR kit (TaKaRa Bio Europe/SAS, France). After heat inactivation at 96°C for 5 min, amplification primers were added to the reaction mixture, and DNA corresponding to positions 5956 to 8535 in isolate HXB2 (GenBank accession numbers K03455 and M38432) was amplified for 20 cycles. Nested PCR in a total reaction volume of 40 μl with Phusion Hot Start II DNA polymerase (Thermo Scientific, Switzerland) was carried out to amplify gp120 (positions 6817 to 7812) and gp41 (positions 7789 to 8382).

(iii) DNA sequencing. Before sequencing, amplified DNA was treated with Illustra ExoStar 1-step reagent (Fisher Scientific, Switzerland). For cycle sequencing, the BigDye Terminator v3.1 cycle sequencing kit (LifeTechnologies, Switzerland) and specific sequencing primers were used. Twenty-five cycles of heat denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and synthesis at 60°C for 4 min were carried out on a 2720 thermal cycler (Life Technologies). Samples were further processed by ethanol precipitation, followed by capillary electrophoresis on a model 3130xl genetic analyzer (Applied Biosystems, Switzerland). The sequences were assembled and edited with SeqMan Pro from the Lasergene 11 package (DNAStar).

Statistical analyses. Statistical analyses were performed using GraphPad Prism 5.04 (GraphPad Software). Data were subjected to either unpaired *t* tests or paired *t* tests, as indicated in the figure legends. The *P* values obtained were considered significant when <0.05. Statistical outlier analysis was performed using the GraphPad Outlier calculator with an alpha of 0.01 (http://graphpad.com/quickcalcs/Grubbs1.cfm).

RESULTS

Transplanting $CD34^+$ cells with partial CCR5 knockdown does not hinder HIV replication. We previously developed a highly efficient microRNA called mirGE that allows efficient knockdown by single-copy transduction (30). In this study, we explored the potential of a mirGE lentivector targeting CCR5 to produce an HIV-resistant immune system in hu mice. The construct consists of a triple hairpin, and the vector cassette contains GFP driven by the same promoter as the miRNA that allows transduced cells to be identified directly. To minimize possible cellular perturbations from multiple vector inserts, we established a protocol that gave us a transduction rate of 20 to 30%. This transduction rate was based on previous work and should correlate with single-copy integration (36). In the first series of experiments, mice were transplanted with mirGE-transduced $CD34^+$ cells without further manipulation (R5 knockdown mice); the $CD34^+$ cells were a mixture of transduced (20 to 30%) and untransduced (70 to 80%) cells (data not shown). As controls, we used mice transplanted with $CD34^+$ cells transduced either with a control GFP lentivector (control-transduced mice) (30) or with untransduced $CD34^+$ cells (untransduced mice). Upon infection with an R5-tropic HIV (YU-2), the percentage and absolute number of GFP-positive $CD4^+$ T cells were increased in R5 knockdown cohorts and not in control-transduced cohorts (Fig. 1a; see also Fig. 3g and h). The $CD4^+$ cell population, however, remained the same over the observation period of 92 days (cohort 1) or showed a $CD4^+$ T-cell loss only at day 134 days (cohort 2) (Fig. 1b). We explain this increase in GFP^+ HIV-resistant $CD4^+$ cells as the result of preferential expansion at the cost of untransduced $CD4^+$ T cells, while the lymphoid system tries to keep the lymphoid T-cell number constant. However, the rates of HIV replication were similar in R5 knockdown mice and control-transduced mice at 92 and 134 days (Fig. 1c). Analysis of absolute numbers of $CD4^+$ T cells (cohort 2) indicated that the control-transduced mice lost $CD4^+$ T cells, whereas $CD4^+$ T cells were more or less maintained in the R5 knockdown mice (data not shown).

In the R5 knockdown mice, CCR5 was downregulated in the GFP-positive $CD4^+$ T cells, but CCR5 was detected on the GFP-negative $CD4^+$ T cells in blood and spleen (Fig. 1d). In the control-transduced mice, CCR5 was detected on GFP-negative and -positive $CD4^+$ T cells. We verified the efficacy of our gene engineering approach by separating transduced from untransduced splenocytes (R5 knockdown mice) by FACS and infected the populations *ex vivo* with R5-tropic HIV. GFP-positive splenocytes had no HIV replication (Fig. 1e).

These results suggest that CCR5 knockdown efficiently protects $CD4^+$ T cells from HIV infection, while CCR5-expressing $CD4^+$ T cells are eradicated. In our mice, despite HIV challenge, at least a proportion of the ~70 to 80% untransduced hematopoietic stem cells survived and continued to produce HIV-permissive $CD4^+$ T cells, which sustained high HIV titers. It is still not entirely clear what effect HIV infection has on $CD34^+$ cells and to what extent they are depleted, if at all (37).

Transplantation of purified CCR5 knockdown $CD34^+$ cells results in mice with "pure" populations of transduced cells *in vivo*. The lack of resistance to HIV infection was likely due to the chimerism of transduced and untransduced $CD34^+$ cells in our initial experiments. Therefore, we sorted the $CD34^+$ cells after transduction into CCR5 knockdown, GFP-positive and -negative fractions obtaining a >90% pure population of GFP-positive $CD34^+$ cells. Mice transplanted with GFP-sorted cells were called FACS-sorted R5 knockdown mice. Analysis of the peripheral blood in six of the FACS-sorted R5 knockdown animals showed a single GFP-positive peak for human $CD45^+$ and $CD4^+$ T cells, suggesting that only transduced $CD34^+$ cells were engrafted in these mice (Fig. 2); the level of GFP-positive cells was a major criterion for successful gene engineering and engraftment. Mice transplanted with the GFP-negative fraction were called FACS-sorted negative mice. They developed $CD4^+$ and $CD8^+$ T-cell populations with no GFP expression (Fig. 2). In contrast, the control-transduced mice had two distinct GFP-negative and -positive populations for $CD45^+$ and $CD4^+$ T cells (Fig. 2). A summary of mice used in this study is pro-

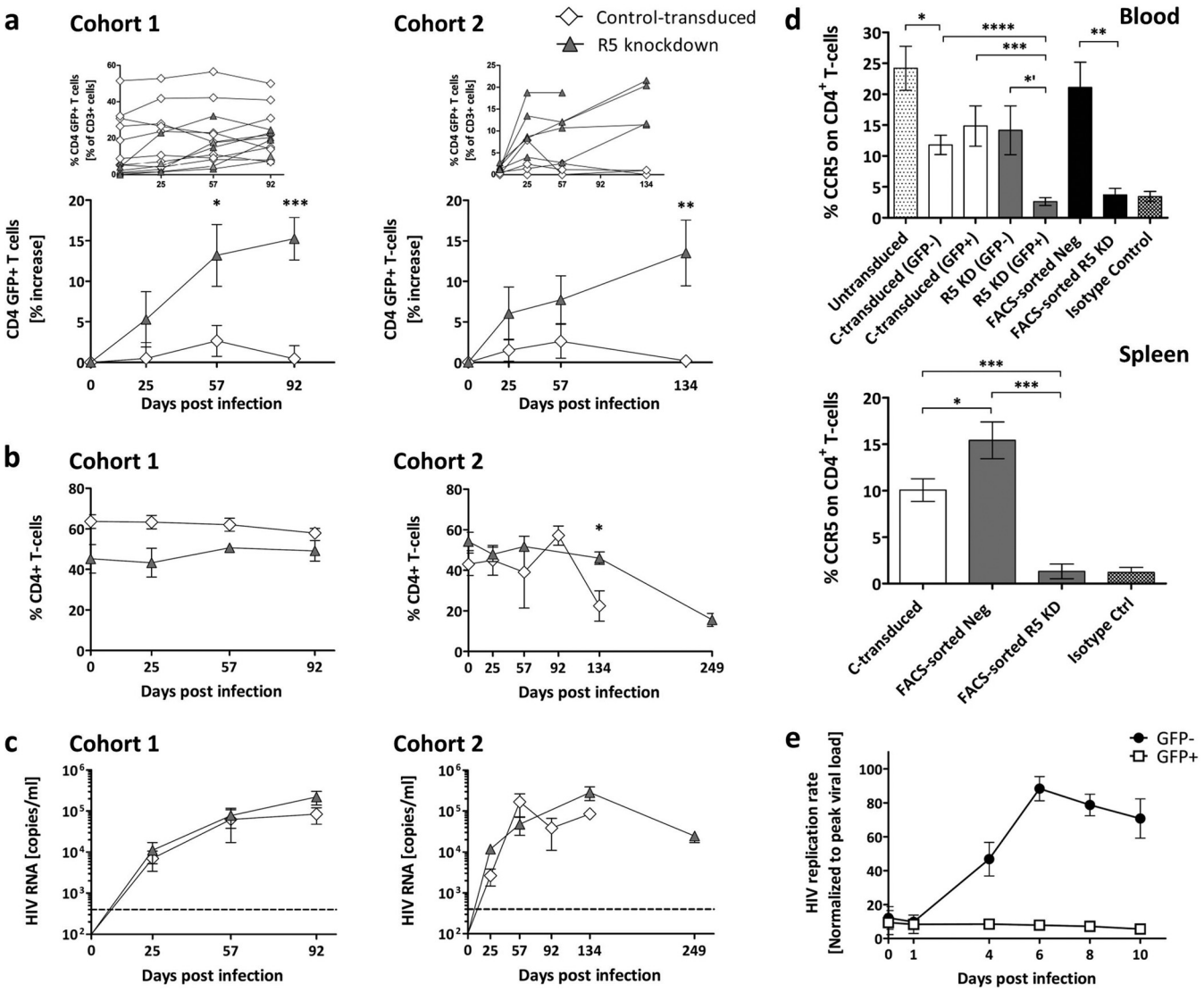

FIG 1 Homeostatic expansion of GFP-positive CD4$^+$ T cells in R5 knockdown mice despite sustained R5-tropic (YU-2) HIV infection. (a) Percent change of GFP-positive CD4$^+$ T cells in the peripheral blood of control-transduced mice (cohort 1, $n = 8$; cohort 2, $n = 4$) and R5 knockdown mice (cohort 1, $n = 6$; cohort 2, $n = 5$). Values are means ± SEMs. *, $P = 0.0152$; **, $P = 0.0034$; ***, $P = 0.0002$. *P* values were determined by two-tailed unpaired *t* test. Insets show the results for individual mice. (b) Frequency of total CD4$^+$ T cells (percentage of total CD3 T cells) for the control-transduced and R5 knockdown cohorts 1 and 2. Values are means ± SEMs. *, $P = 0.0146$. *P* values were determined by two-tailed unpaired *t* test. (c) HIV RNA copies per milliliter of blood plasma collected for the control-transduced and R5 knockdown cohort 1 and cohort 2 over 92 and 134 days, respectively. Time of termination was chosen at random for the various groups. The dashed line indicates 400 copies/ml, the detection limit of the HIV RNA assay. Values are means ± SEMs. (d) Percentage CCR5 expression on total CD4$^+$ T cells in peripheral blood and spleens of various cohorts of mice. Values are means ± SEMs. For blood, asterisks indicate *P* values as follows: *, $P = 0.0237$; *′, $P = 0.0121$; **, $P = 0.0035$; ***, $P = 0.0007$; ****, $P = 0.0001$. For spleens, asterisks indicate *P* values as follows: *, $P = 0.041$; ***, $P = 0.0003$. *P* values were determined by two-tailed unpaired *t* test. (e) HIV replication is inhibited *ex vivo* in GFP-positive sorted splenocytes from R5 knockdown mice. Splenocytes were isolated from R5 knockdown mice 20 weeks after CD34$^+$ cell injection. Splenocytes were sorted into GFP-positive ($n = 5$) and GFP-negative ($n = 5$) fractions at 99% purity and challenged with R5-tropic HIV (YU-2). The amount of HIV production in the culture supernatant was monitored by HIV p24 ELISA. Viral loads were normalized to the peak viral load under each condition (means ± SEMs).

vided in Table 1. Mice with less than 5% human engraftment in the peripheral blood before HIV infection were excluded.

Transplanting purified CCR5 knockdown CD34$^+$ cells dramatically lowered viral load and protected HIV target cells *in vivo*. The FACS-sorted R5 knockdown mice had markedly lower viral loads than the FACS-sorted negative mice over 28 weeks (Fig. 3a). Peak viremia for the FACS-sorted R5 knockdown mice was, on average, 4.2×10^3 copies/ml, and FACS-sorted negative mice had 3.5×10^5 copies/ml. Viral loads for the untransduced, control-transduced, R5 knockdown, and FACS-sorted negative mice were similar. The FACS-sorted R5 knockdown mice had lower viral loads than all cohorts.

CD4$^+$ T cells (percentage of total CD3$^+$ T cells) from the FACS-sorted negative mice declined steadily upon infection (day 0, 55%; day 134, 20%) (Fig. 3b). In contrast, the FACS-sorted R5 knockdown mice showed a steady increase in CD4$^+$ T cells (day 0, 33%; day 196, 65%). Furthermore, the absolute numbers of CD4$^+$ T cells increased for the FACS-sorted R5

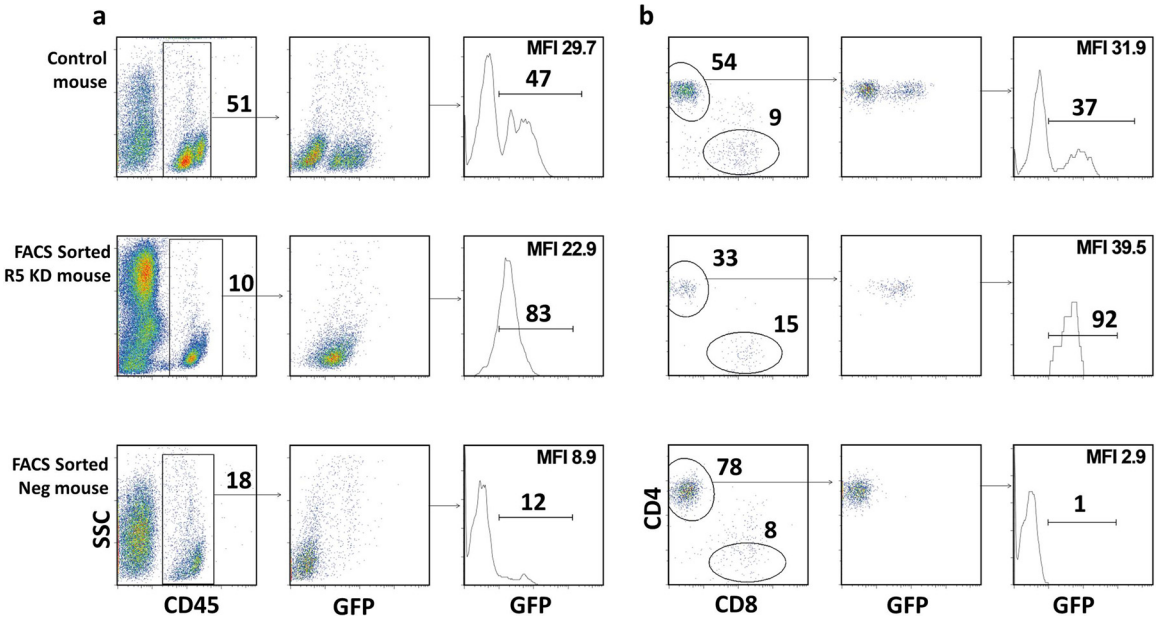

FIG 2 GFP-positive CCR5 knockdown sorted CD34$^+$ HSPCs produced mice with "pure" populations of transduced cells *in vivo*. (a) FACS plots showing the percent human engraftment of CD45$^+$ (percentage of live cells) and GFP-positive CD45$^+$ (percentage of human CD45) cells for representative control-transduced, FACS-sorted R5 knockdown, and FACS-sorted negative mice before HIV infection. (b) FACS plots showing the percentage CD4$^+$ T cells (percent CD3$^+$), CD8$^+$ (percent CD3$^+$), and GFP-positive CD4$^+$ T cells (percent CD4$^+$) in the peripheral blood before HIV infection of the same mice as in panel a. For all cell subset analyses, the subgating was done as follows: total live cells and CD45$^+$, CD3$^+$, CD4$^+$, and CD8$^+$ cells. Mean fluorescence intensity (MFI) for GFP is indicated.

knockdown mice but decreased for the FACS-sorted negative mice (data not shown).

At euthanasia, for the FACS-sorted R5 knockdown mice, 70% of CD4$^+$ T cells in the blood and 83% of CD4$^+$ T cells in the spleen were GFP positive (Fig. 3c and d). In the control-transduced and R5 knockdown groups, the values were, on average, 20% and 18% in the blood and 21% and 20% in the spleen, respectively (Fig. 3c and d). Similarly, the CD4$^+$/CD8$^+$ T-cell ratios in the blood (end/preinfection) and spleen were very low in the various groups except for the FACS-sorted R5 knockdown group (Fig. 3e and f). Absolute numbers of GFP-positive CD4$^+$ T cells expanded significantly upon HIV challenge in the FACS-sorted R5 KD mice (Fig. 3i), and CCR5 expression was downregulated in the blood and spleens of the FACS-sorted R5 knockdown mice (Fig. 1d).

In summary, transplantation of GFP-positive sorted CD34$^+$ cells produced mice with high levels of gene-engineered CCR5 knockdown CD4$^+$ T cells *in vivo*. This resulted in long-term inhibition of HIV replication *in vivo* and preservation of HIV target cells in the blood and spleen.

Outlier analysis and follow-up of mice that did not meet acceptance criteria. The FACS-sorted R5 knockdown mice typically controlled the virus long term (titers $< 10^4$ copies/ml) while maintaining a high level of GFP-positive CD4$^+$ T cells in the blood (Fig. 4a). In contrast, a single FACS-sorted R5 knockdown mouse (number 954) showed an unexpected decline in GFP-positive CD4$^+$ T cells with high HIV copy numbers, $>10^5$ copies/ml, on days 57 and 92 (Fig. 4a). We performed an outlier analysis (see Materials and Methods), and at four different times, viral loads detected in mouse 954 were statistical outliers (Fig. 4a). Based on this, mouse 954 was not included in the mean values and was analyzed separately (see below). At termination, this mouse had much lower splenic engraftment of total human splenocytes, with 31% human cells, than did the FACS-sorted R5 knockdown mice, which had 54% (standard error of the mean [SEM], ±11%). GFP-

TABLE 1 Mice used in this study

Trait	% GFP$^+$ CD34$^+$ cells pretransplantation	Total no. of mice	No. of mice with:			
			% engraftment (>5% hCD45$^+$)	% GFP$^+$ CD4$^+$ T cells (>70%)	% GFP$^+$ CD4$^+$ T cells (20–70%)	% GFP$^+$ CD4$^+$ T cells (<20%)
Untransduced	NA[a]	15	11	NA	NA	NA
Control transduced	15–40	17	12	0	5	7
R5 knockdown	17–20	15	11	0	0	11
FACS GFP positive[b]	12–32	20	10	6	4	0
FACS GFP negative	NA	26	15	NA	NA	NA

[a] NA, not applicable.
[b] FACS GFP-positive cells prior to FACS cell sorting for GFP-positive cells.

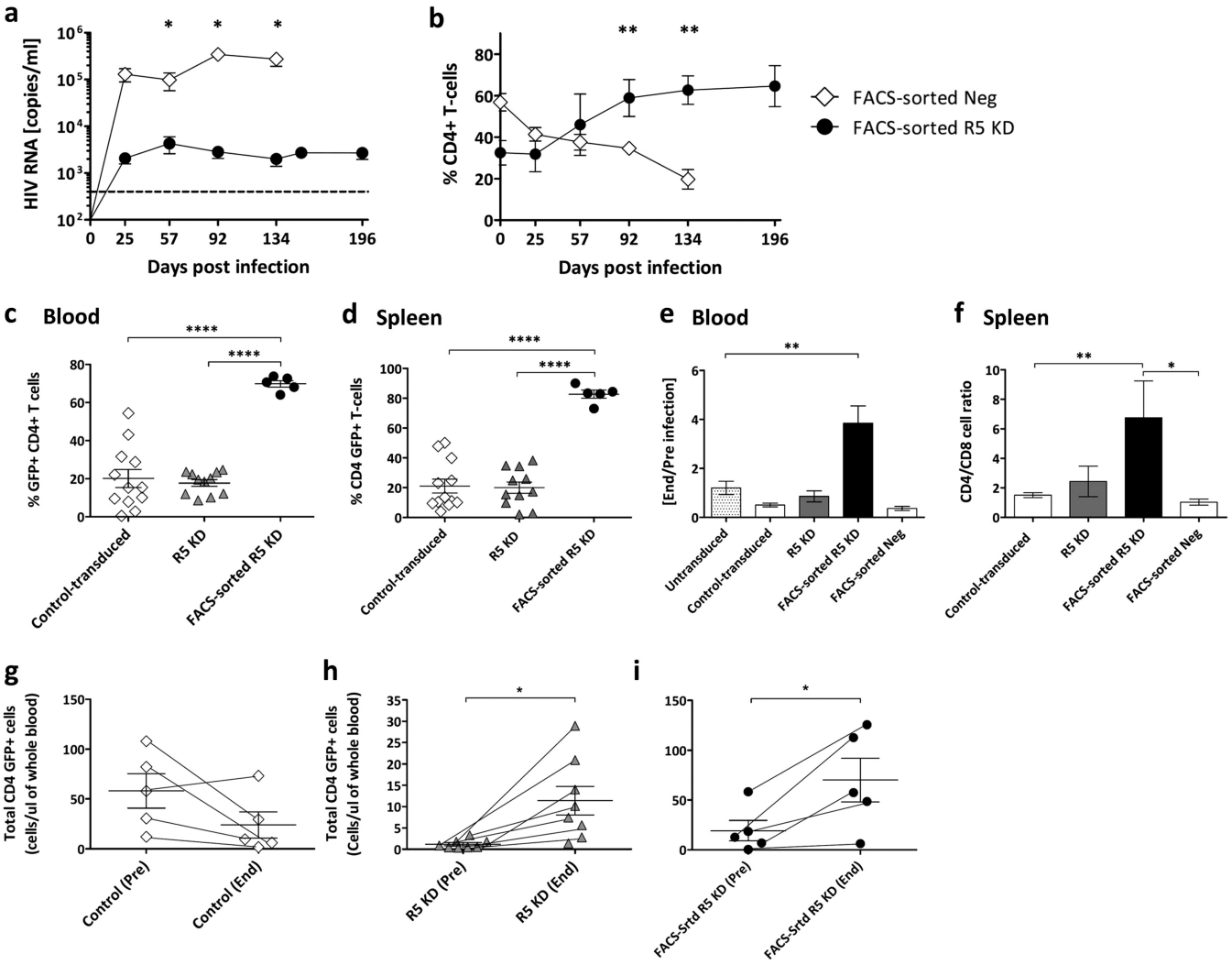

FIG 3 Sustained HIV load inhibition in FACS-sorted R5 knockdown mice. (a) HIV RNA copies per milliliter in blood plasma from FACS-sorted R5 knockdown (YU-2 [$n = 5$]) and FACS-sorted negative ($n = 15$ [YU-2, $n = 9$, and JRCSF, $n = 6$]) mice collected over 134 and 196 days, respectively. The viral load detection limit is indicated by the dashed line (400 copies/ml). Values are means ± SEMs. Single asterisks indicate P values of 0.0486, 0.0188, and 0.0391 for days 57, 92, and 134, respectively. P values were determined by a two-tailed unpaired t test. (b) Percent $CD4^+$ T cells of FACS-sorted R5 knockdown ($n = 5$) and FACS-sorted negative ($n = 15$) mice. Values are means ± SEMs. Double asterisks indicate P values of 0.0017 and 0.0018 for days 92 and 134, respectively. P values were determined by two-tailed unpaired t test. (c) Percent GFP-positive $CD4^+$ T cells in the peripheral blood at termination. Values are means ± SEMs. ****, $P = 0.0001$. P values were determined by two-tailed unpaired t test. (d) Percent GFP-positive $CD4^+$ T cells in the spleen at termination. Values are means ± SEMs. ****, $P = 0.0001$. P values were determined by two-tailed unpaired t test. (e) Change of $CD4^+/CD8^+$ T-cell ratio in the peripheral blood, comparing the $CD4^+/CD8^+$ T-cell ratio of each cohort end and preinfection. Values are means ± SEMs. **, $P = 0.0026$. P values were determined by two-tailed unpaired t test. (f) $CD4^+/CD8^+$ T-cell ratio at termination in the spleens of various cohorts of mice. Values are means ± SEMs. **, $P = 0.0059$; *, $P = 0.039$. P values were determined by two-tailed unpaired t test. (g to i) Absolute numbers of GFP^+ $CD4^+$ T cells or total $CD4^+$ T cells/microliter of blood of representative mice from the control-transduced ($n = 5$), R5 knockdown ($n = 8$), and FACS-sorted R5 knockdown ($n = 5$) cohorts are shown. Values are means ± SEMs. Single asterisks in panels h and i indicate P values of 0.0194 and 0.0369, respectively. P values were determined by paired t test.

positive $CD4^+$ T cells were barely detected in the spleen of mouse 954 (6%), while the FACS-sorted R5 knockdown mice had, on average, 83% (SEM, ±2%) GFP-positive $CD4^+$ T cells in the spleen (Fig. 3d).

The inclusion criterion we defined as successful reconstitution for FACS-sorted R5 knockdown mice was 70% GFP^+ $CD4^+$ T cells in the peripheral blood before infection. Four mice did not meet this criterion despite being transplanted with GFP-positive sorted $CD34^+$ cells (Fig. 4b). Mouse 1113 had no protection against HIV and had a limited expansion of GFP-positive $CD4^+$ T cells (23% on day 0 to 43% on day 137). Mice 958 and 1115 had massive expansions of GFP-positive cells, reaching close to 100% of the total $CD4^+$ T-cell population, which went in parallel with a decrease in the viral load (Fig. 4b). For mouse 1608, the dynamics of recovery of GFP-positive $CD4^+$ T cells and viral load were slower, and recovery of GFP-positive $CD4^+$ T cells on day 134 was less extensive (Fig. 4b). These three mice (958, 1115, and 1608) maintained a high CD4/CD8 ratio, and the percentage of GFP-positive $CD4^+$ T cells in the blood increased upon infection from 24 to 80, 39 to 82, and 37 to 52%, respectively. These animals also had high frequencies of GFP-positive $CD4^+$ T cells in the spleens, i.e., 59, 60, and 83%, respectively.

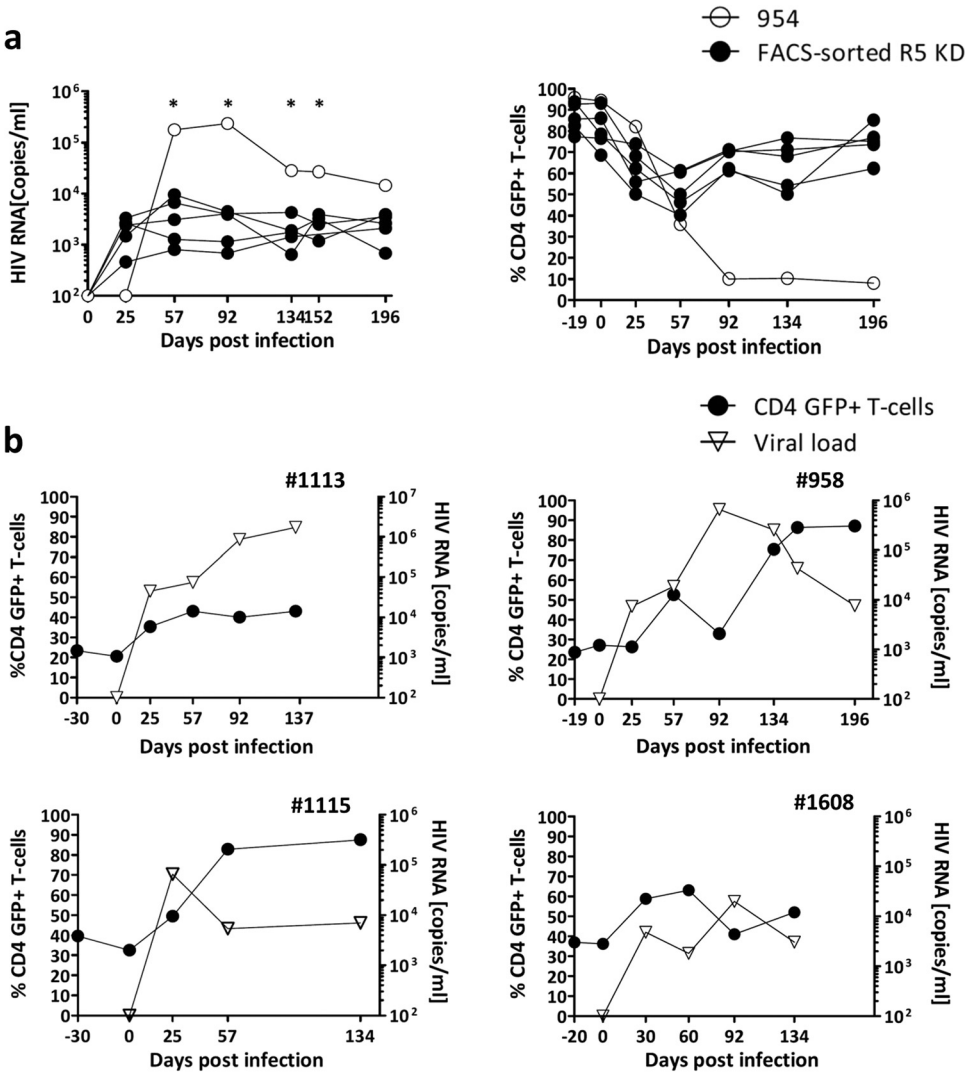

FIG 4 Outlier analysis and examples of viral load control due to homeostatic expansion of transduced cells. (a) Left graph, viral load of the FACS-sorted R5 knockdown mice (YU-2) and viral load of outlier FACS-sorted R5 knockdown mouse 954 (YU-2). Right graph, percent GFP-positive $CD4^+$ T cells of the FACS-sorted R5 knockdown mice and mouse 954. Percent GFP-positive $CD4^+$ T cells is shown as a percentage of total $CD4^+$ T cells. *P* values were determined by GraphPad Outlier calculator. *, $P < 0.001$. (b) Viral load and percent GFP-positive $CD4^+$ T cells of four individual FACS-sorted R5 knockdown mice (mouse 1113, JRCSF; mouse 958, YU-2; mouse 1115, JRCSF; and mouse 1608, JRCSF) that were excluded due to not reaching the inclusion criteria of >70% GFP-positive $CD4^+$ T cells before HIV infection.

Strikingly, we observed this level of expansion (Fig. 4b) of HIV-resistant GFP-positive $CD4^+$ T cells and a concomitant inhibitory effect on HIV only in mice transplanted with CCR5 knockdown GFP-sorted $CD34^+$ cells (these animals had 30% [±4% SEM] GFP^+ $CD4^+$ T cells on day 0). This degree of expansion was not seen in R5 knockdown mice (which had 5% ± 2% GFP^+ $CD4^+$ T cells before HIV infection). Based on these results, we estimate that at least 20% of $CD4^+$ T cells need to be CCR5 repressed to observe homeostatic expansion and relevant effects on viremia.

Preserved engraftment and preferential expansion of central memory T cells in FACS-sorted R5 knockdown mice upon HIV infection. Engraftment as reflected in peripheral blood decreased in all control cohorts but increased in the FACS-sorted R5 knockdown mice (Fig. 5a). This effect on total engraftment was even more impressive in the spleen. FACS-sorted R5 knockdown mice had 10 times more human cells than control cohorts (Fig. 5b).

We evaluated the $CD4^+$ and $CD8^+$ effector ($CD45RA^{pos}$ $CCR7^{neg}$), effector memory ($CD45RA^{neg}$ $CCR7^{neg}$), naive ($CD45RA^{pos}$ $CCR7^{pos}$), and central memory ($CD45RA^{neg}$ $CCR7^{pos}$) T-cell subsets in the blood and spleens of the FACS-sorted R5 knockdown and representative FACS-sorted negative mice (Fig. 5c to f). In the peripheral blood of the FACS-sorted negative mice, the frequency of central memory $CD4^+$ T cells was significantly decreased, and the $CD8^+$ central memory T-cell subset was unchanged (Fig. 5c and e). In contrast, central memory $CD4^+$ and $CD8^+$ T cells were increased in the FACS-sorted R5 knockdown mice. Similarly, more $CD4^+$ and $CD8^+$ central memory T cells were present in the spleens of the FACS-sorted R5 knockdown mice (Fig. 5d and f). We observed no differences between the cohorts for effector and effector memory cells (data not shown). Notably, however, there was a trend toward a decrease in naive $CD4^+$ and $CD8^+$ T cells in all cohorts (data not shown).

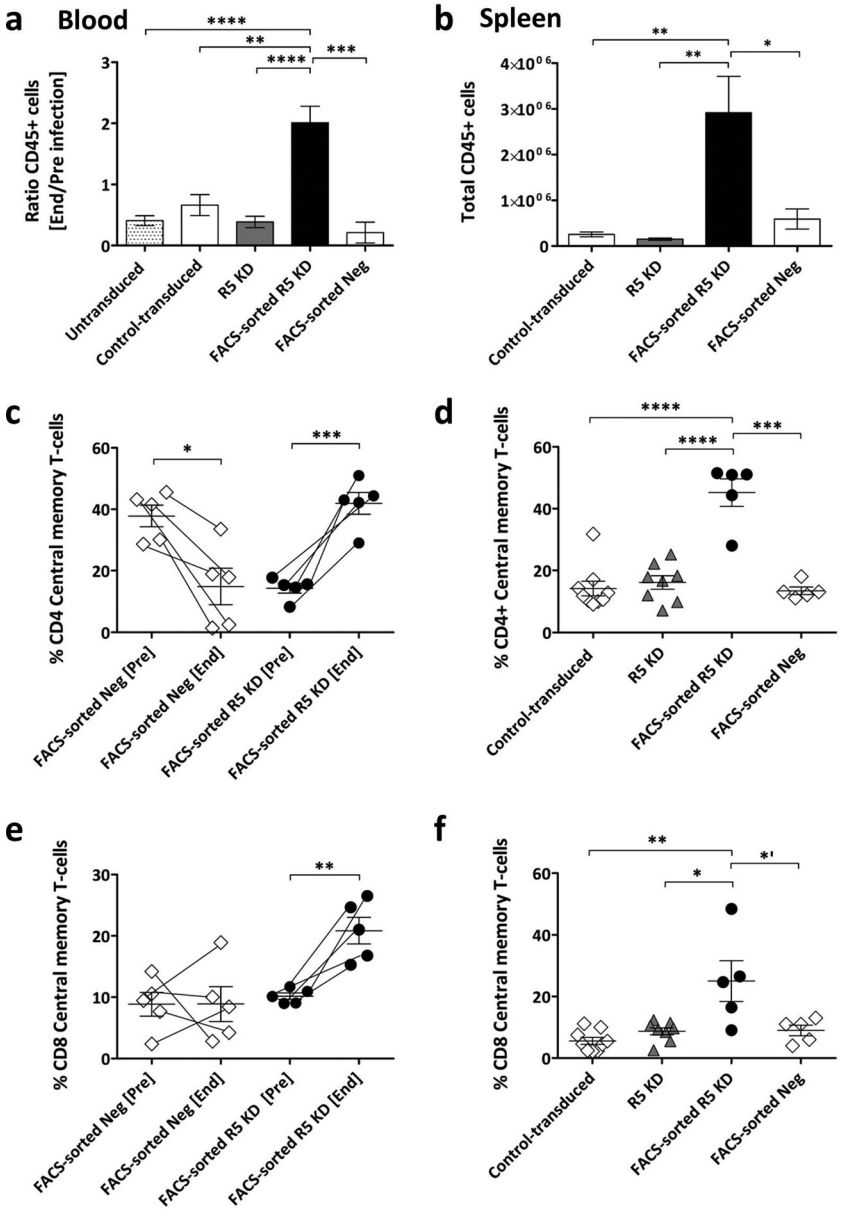

FIG 5 Increased engraftment and central memory T cells in blood and spleens of FACS-sorted R5 knockdown mice. (a) Change in level of peripheral blood engraftment expressed as the ratio of total $CD45^+$ at the end and preinfection. Values are means ± SEMs. **, $P = 0.0031$; ***, $P = 0.0006$; ****, $P < 0.0001$. *P* values were determined by two-tailed unpaired *t* test. (b) Absolute numbers of human cells ($CD45^+$) in the spleen at termination. Values are means ± SEMs. *, $P = 0.0229$; **, $P < 0.0019$. *P* values were determined by two-tailed unpaired *t* test. (c and e) Percent $CD4^+$ and $CD8^+$ central memory T cells in the peripheral blood, preinfection and at the end time point. Values are means and SEMs. *, $P = 0.0153$; **, $P = 0.0042$; ***, $P = 0.0004$. *P* values were determined by paired *t* test. (d and f) Percent $CD4^+$ and $CD8^+$ central memory T cells in the spleen at termination. Values are means ± SEMs. *′, $P = 0.0472$; *, $P = 0.0106$; **, $P = 0.0022$; ***, $P = 0.0001$; ****, $P < 0.0001$. *P* values were determined by two-tailed unpaired *t* test.

Evidence of shift from R5- to X4-tropic strain in one mouse. As described above (Fig. 4), FACS-sorted R5 knockdown mice had a low viral load and maintained high levels of CCR5 knockdown $CD4^+$ T cells. Mouse 954 was clearly an outlier: it had high viral titers and lost GFP-positive $CD4^+$ T cells (Fig. 6a). We hypothesized that this mouse might have had a tropism shift of the virus from R5 to X4. We performed HIV population sequencing (from plasma) on days 57 and 196. As a control, we analyzed mouse 958 (Fig. 6b). Sequencing revealed no mutations in mouse 958. Mouse 954 had mutations in the V3 loop of the HIV envelope sequence (Fig. 6c), resulting in amino acid substitutions to basic amino acids as indicated (Fig. 6d). Substitutions in the V3 loop to basic amino acids have been reported to result in a switch from R5 to X4 tropism (38).

DISCUSSION

In this study, we investigated a lentiviral vector-based, CCR5-targeting miRNA as a tool for engineering an HIV-resistant human immune system. We show that (i) the miRNA-based vector was very efficient in downregulating CCR5 on T cells and prevented their infection by HIV *ex vivo*, (ii) only mice that were trans-

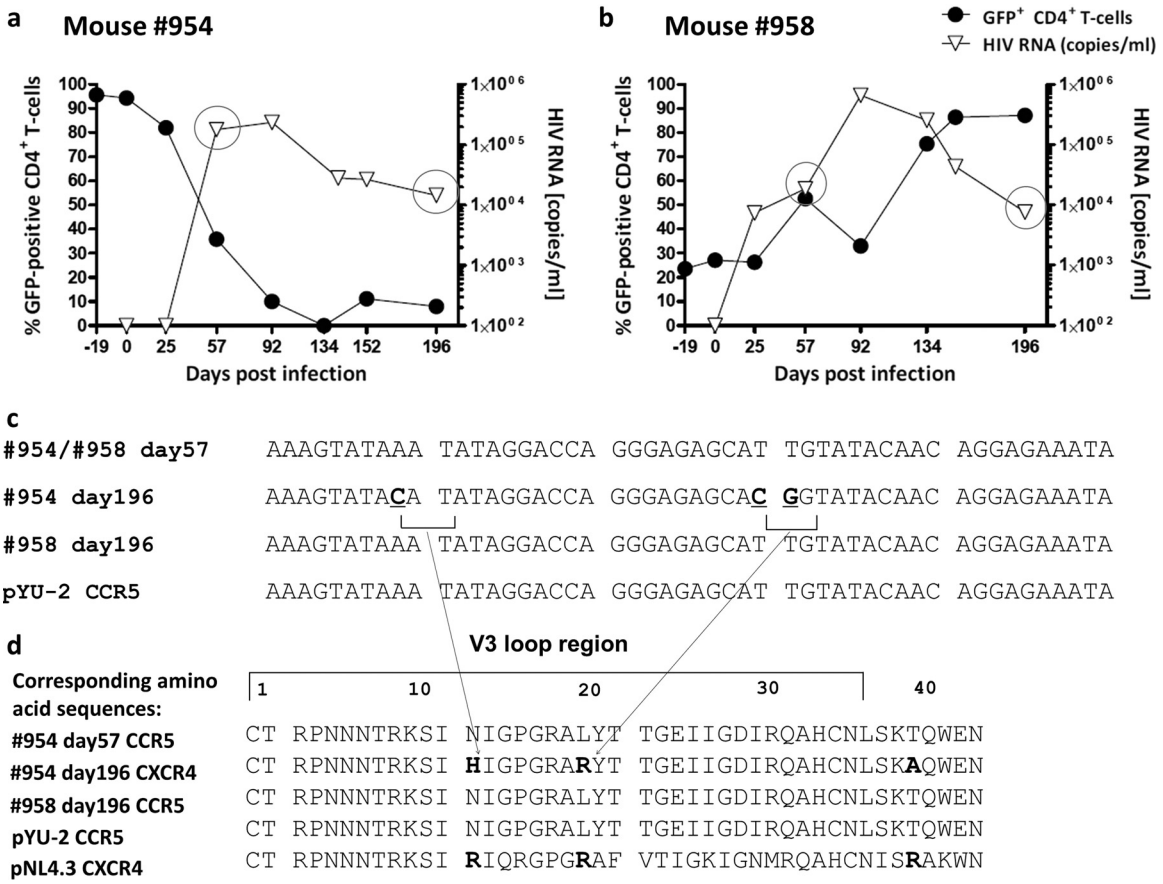

FIG 6 Population sequencing of HIV in plasma: evidence for gene therapy failure. (a and b) Plots showing the percent GFP-positive CD4$^+$ T cells in peripheral blood (as a percentage of CD3$^+$ T cells) on the left *y* axis and HIV load on the right *y* axis for mice 954 and 958, respectively. Mouse 954 had a high and sustained viral load over time, with a complete loss of GFP-positive CD4$^+$ T cells. Mouse 958 experienced an expansion of GFP-positive CD4$^+$ T cells from less than 30% on day 19 to more than 80% on day 196. (c) On day 57, both animals had a homogenous HIV population in the peripheral blood; sequencing data of HIV envelope V3 loop are consistent with an R5-tropic HIV strain. On day 196, mouse 954, which experienced a complete loss of GFP-positive CD4$^+$ T cells (blood and spleen), had detectable mutations within the V3 loop of HIV. For mouse 958, no mutations were detected in the V3 loop on day 196, indicating the presence of a homogenous HIV population. (d) Changes to basic amino acids H and R. According to a Geno2Pheno (http://www.geno2pheno.org/) analysis of the obtained sequence in panel c, there is only 18% confidence that the virus at day 196 of mouse 954 was not an X4 variant.

planted with a preselected population of transduced CD34$^+$ cells and maintained gene-engineered CD4$^+$ T cells had a dramatically reduced viral load (functional cure), and (iii) the HIV-infected mice transplanted with miRNA CCR5 gene-engineered CD34$^+$ cells showed a dramatic expansion of memory T cells (i.e., the miRNA-edited T cells were mainly of this phenotype). Thus, we provide here preclinical proof of concept for gene engineering of an HIV-resistant immune system through the use of vector-mediated miRNA expression and the need for a certain threshold of gene-engineered CD34$^+$ cells for functional cure of HIV.

While gene engineering of HIV-resistant cells is a viable option for cure of HIV, major issues remain to be solved. These include finding the best antiviral moiety or combination, the most efficacious way to gene engineer the CD34$^+$ cells, and the threshold of gene-engineered CD34$^+$ cells needed for functional cure.

Lentivirus-based transduction has been supplemented by gene-targeting methods, such as ZNF or Talen nucleases, or the Crispr/Cas system for gene editing (23–25). However, off-target effects of these methods are still unknown, and gene engineering in primary cells is only modestly effective (26). And even though no adverse events have been reported, there is less experience in clinical trials with gene-targeting methods than with lentivirus-based transduction. Thus, we opted for lentivirus-based gene engineering (39–41). Furthermore, we are the first to engage in miRNA technology for knocking down the HIV coreceptor CCR5 in CD4$^+$ T cells via gene engineering of CD34$^+$ cells. miRNAs closely mimic naturally occurring pri-miRNAs and thus are less likely to cause oversaturation of the RNA interference pathway and to affect cellular homeostasis than the widely used shRNAs (42, 43). However, miRNAs are thought to have a lower capacity to downregulate target genes than shRNAs. In this study, we used a miRNA we developed with optimized features that efficiently knock down target genes upon single-copy vector transduction (30). *Ex vivo*-sorted cells were indeed resistant to a challenge with CCR5-tropic strains. However, bulk transplantation of transduced CD34$^+$ cells into mice resulted in a human lymphoid system that replicated HIV similarly to untransduced hu mice but preserved CD4$^+$ T-cell counts. Similar data have been reported previously (15).

We hypothesized that the majority of HSPCs needs to be gene engineered to see an effect on the HIV load; otherwise, the HIV-permissive CD4$^+$ T cells that originated from the untransduced

$CD34^+$ cells would "outnumber" the HIV-resistant cells. Thus, we used GFP to allow for efficient sorting of transduced $CD34^+$ cells before transplantation. By doing so, we found that to achieve long-term suppression of viral load, more than 70% of $CD34^+$ transplanted should be gene engineered. Walker et al. obtained an average engraftment (± standard deviation [STD]) level of anti-HIV vector-transduced cells of 17.5% ± 8% in the peripheral blood and argued that these numbers of cells were insufficient to see any decrease in plasma viremia (15). Furthermore, a very recent publication from the same group sorted the gene-engineered $CD34^+$ cells with a truncated version of CD25 (tCD25) before their transplantation into 2- to 5-day-old NRG mice (20). They found that mice transplanted with tCD25-purified $CD34^+$ cells had normal multilineage hematopoiesis, similar to mice transplanted with untransduced $CD34^+$ cells. Upon HIV challenge, tCD25-transplanted mice did not suffer from HIV-induced $CD4^+$ T-cell depletion as did the untransduced mice, and tCD25 mice had a 1.5-log inhibition in plasma viremia compared to that of mice with untransduced $CD34^+$ cells. Our data nicely complement the data provided by Walker et al. and Barclay et al. and underline the importance of the number of transduced cells that are required for efficient HIV gene therapy. Notably, three hu mice transplanted with purified gene-engineered $CD34^+$ cells showed at baseline ~30% GFP^+ cells which expanded substantially upon HIV infection; the expansion went along with a decrease in viral load. The data for these three mice were reminiscent of the data reported by Holt et al. showing that disruption of CCR5 by zinc finger nucleases was achieved in ~20% of $CD34^+$ cells and resulted in viral repression over time (16).

Obviously, in humans, GFP-based sorting would not be an option, given the xenogeneic nature of the protein. However, novel strategies for sorting of transduced $CD34^+$ cells based on the expression of truncated cellular surface receptors, such as CD25 (20), the epidermal growth factor receptor (44), or the nerve growth factor receptor (45), are very promising for achieving high numbers of engrafted gene-engineered cells. An alternative approach to pretransplantation sorting would be *in vivo* selection of transduced cells (46, 47). Regrettably, current *in vivo* selection methods use potentially carcinogenic compounds, such as mycophenolate, methotrexate, or alkylating agents (i.e., O^6-benzylguanine/bis-chloroethylnitrosourea), that offset their use in a disease, such as HIV, that is amenable to an efficient and well-tolerated cART. We want to emphasize that in our gene-engineering efforts, we aimed for single lentiviral copy integration. The two recent phase I clinical trials used gene engineering protocols that resulted in vector copy numbers ranging from 2 to 4 per genome of bone marrow cells prior to transplantation without documenting any adverse events over an observation period of >20 months (39, 40). Thus, ensuring $CD34^+$ transduction might present another alternative for increasing the number of gene-engineered $CD34^+$ cells. These protocols appeared not to affect the long-term engraftment negatively in these phase I clinical trials.

In fact, we do not know the number of gene-engineered HSPCs needed to render the immune system resistant to HIV. As outlined above, we aimed for a rather pure population of gene-engineered HSPCs as proof of preclinical concept. However, we observed HIV-lowering effects in some mice with 20 to 40% engraftment of transduced cells, data similar to those reported by Holt et al. (16). HIV, certainly by killing untransduced cells via its cytopathic effects, will promote the expansion of HIV-resistant cells. To what extent the HIV-resistant cells will foster an efficient HIV-specific immune response and thereby constrain HIV remains unknown. Whether additional factors contribute to HIV-lowering effects remains unknown.

White blood cell counts from HIV-infected mice generated with FACS-sorted R5 KD cells showed an expansion of central memory $CD4^+$ and $CD8^+$ T cells, while all other groups showed a progressive loss of these $CD4^+$ memory T cells and no change in $CD8^+$ T cells. This pattern was also evident when looking at the splenocytes. These memory $CD8^+$ T cells might have contributed to the control of HIV. There was a decrease in the frequency of naive $CD4^+$ and $CD8^+$ T cells in the peripheral blood in both FACS-sorted negative and FACS-sorted R5 KD mice, whereas in the spleen, the naive $CD4^+$ and $CD8^+$ T cells in the FACS-sorted R5 KD mice tended to be higher (data not shown). The expansion of central memory T cells is reminiscent of the immune reconstitution seen in HIV-infected patients on ART (48).

HIV is known for its high mutational rate. In this respect, we observed one mouse (mouse 954) with an escape mutation. Despite high levels (day 0) of engraftment of CCR5 knockdown cells, this mouse had a high viral load and a complete loss of circulating $CD4^+$ T cells. Population sequencing of the V3 loop indicated a likely shift to X4 tropism, which might explain the uncontrolled infection. The mutations were detectable in the blood only at relatively late time points. This might be due to an initial compartmentalized replication of the X4-tropic strains in the thymus. We previously showed that X4-HIV NL4-3 severely depleted the thymus, whereas YU-2 had only minor effects (49). However, we do not know whether the potential emergence was due to the CCR5 knockdown or was just a coincidence. Indeed, emergence of CXCR4-tropic strains may occur without any immune or drug pressure in hu mice infected with CCR5-tropic strains (50). In any case, CCR5 knockdown should be done in concert with another strategy to constrain HIV (i.e., including another anti-HIV moiety, combining with efficient antiretrovirals, or boosting the immune response in parallel to transplantation). Indeed, the solidness of successful gene engineering by the expression of more than one antiviral moiety may prevent HIV evolution (51). Gene engineering could be combined with conventional ART: combining two treatment modalities was efficient in cell lines (52), as induction therapy (53) or with anti-PD1 antibodies that decrease viral load and increase the level of $CD4^+$ T cells in HIV-infected mice (54). In any case, gene engineering efforts cannot promote more virulent HIV strains, either for the individual patient or for the general population.

In summary, our results provide the first preclinical proof of concept that transplantation of miRNA CCR5 knockdown $CD34^+$ cells can lead to long-term control of HIV viremia. Translation of our results to the clinical setting is relatively straightforward but will require the implementation of existing strategies for pre- and posttransplantation selection compatible with human use. At this point, our strategy demonstrates long-term viral control but not yet a cure. However, while a cure remains the ultimate goal, long-term viral control independent of antiretrovirals is a relevant intermediate step, worth translating to the clinical setting. We believe that a definitive cure of HIV might indeed come from a combination of different approaches such as CCR5 knockdown combined with drug therapy, vaccination, or a second gene therapy target.

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R.M. designed and performed experiments, analyzed data, and wrote the paper. R.F.S. and K.-H.K. designed the experiments, supervised the work, analyzed the data, and wrote the paper. M.S.P. was involved in the initial conceptualization of the study, supervised aspects of the work, and helped to write the paper. A.A. helped to coordinate the work with the mice and transplantation of newborn mice, process cord blood, and write the paper. P.S. helped supervise aspects of the work and designed certain experiments. V.J. helped to write the paper and analyze data. G.G.-H., D.L., and M.-A.R. helped to terminate experiments and process cord blood samples. S.I. helped in processing of cord blood samples. S.R. provided the expertise and obtained the viral loads for HIV-infected mice. M.G.M. gave highly valuable input in the entire study.

We declare no conflicts of interest.

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4. Discussion

4.1 Identification of potential IFN-stimulated HIV-1 restriction factors

The main aim of my thesis was to identify whether additional cellular host restriction factors, which are anti-HIV active, exist, and if so, to characterize their molecular mechanism(s) of action. It has been previously shown that IFN-α treatment blocks HIV-1 replication in CD4+ T cell lines[102, 103], in the monocytic cell line THP-1, as well as in macrophages[104], and that most HIV-1 restriction factors identified so far are known ISGs[105-107]. Thus, we assumed that additional RFs would also be ISGs and aimed doing a more detailed analysis of the genes upregulated by IFN-α treatment that might be causal for HIV-1 inhibition.

For that purpose, we did NGS to identify potential RFs subsequent to IFN-α treatment in cells where HIV-1 was clearly inhibited by IFN-α. We first tested various cell lines for their IFN-responsiveness - among the cell lines screened, MT4R5 and SupT1R5 cells showed the highest responsiveness to IFN-α as documented by the upregulation of some prototypical ISGs. Of note, MT4R5 and SupT1R5 cells lack APOBEC3G and Tetherin, which might mask the potential effect of other restriction factors. In any case, we infected the cells with wild-type HIV-1: the HIV accessory genes, Vif and Vpu, present in the wild-type HIV-1, should have been able to counteract APOBEC3G and Tetherin even if some residual proteins subsequent to IFN-α treatment would have been present. In the MT4R5 cells IFN-α resulted in a clear dose-dependent and persistent reduction of newly produced HIV-1 virions, while in SupTR5 cells this effect was less pronounced and mainly present only at day 3 after infection. IFN-α apparently interfered with HIV-1 during reverse transcription and integration of the HIV-1 genome into the host chromosome since intermediate reverse transcription products and proviral DNA was substantially lower in IFN-treated cells. Thus, potential IFN-induced RF act at the pre-integration complex, transport into the nucleus or the integration process itself. Our results are consistent with previous reports, performed in primary macrophages, the monocytic cell line THP-1[104], and CD4+ T-cells[108], where IFN-α blocks HIV-1 infection between reverse transcription and integration. IFN-α may also be anti-HIV active at later stages in the HIV-1 replication cycle, but the assays we were using, did not allow searching for it.

We argued that the genes responsible for the IFN-α mediated inhibition of HIV-1 in MT4R5 and SupT1R5 cells would be the same, although the antiviral effect of IFN-α was only transient in SupT1R5 cells. Thus, we determined what genes were upregulated in MT4R5

and SupT1R5 treated with IFN-α. For this, we did NGS of MT4R5 and SupT1R5 cells treated with IFN-α for 6 hours – we choose this time point since within this time frame the first wave of gene upregulation takes place[109] and we did not want to have confounding results due to 2nd or 3rd waves upregulation of genes. We assumed that the first wave of genes upregulated contains potential RFs, and not the 2nd or 3rd wave, but this assumption might be wrong. We obtained a list of 162 candidate genes, which were upregulated at once in both cell lines. To further characterise the relevance of the 162 candidates, we performed a gene ontology analysis by looking at the biological processes, the enriched pathway maps and the process networks. All three approaches provided similar results - not very surprisingly, the 162 genes were enriched in the immune response and antiviral response pointing to an antiviral state triggered by IFN-α. We focused on the pathway maps, since it provides a more stringent list of genes potentially important against HIV-1 replication and it helps us to define a selected number of genes as opposed to the biological processes and process networks that provide a large list of candidate genes.

The pathway maps analysis provided an enrichment of relevant genes, in particular 6 genes with a previously reported anti-HIV activity (OAS1, OAS2, OAS3[110], ISG15[111, 112], PKR (EIF2AK2)[113] and USP18[114]) and 7 genes with previously reported antiviral activity (IFI6[115], IRF7[116, 117], ISG54 (IFIT2)[118], MDA-5 (IFIH1), RIG-I (DDX58)[117, 119], MX1[120] and NMI[121]). The identification of the 6 genes with anti-HIV activity and anti-viral activity validates our approach of using NGS for the identification of anti-HIV restriction factors. Of note, the most "famous" RF, APOBEC3G, Tetherin/Bst-2, SAMHD1, TRIM5α and the recently identified MX2[122, 123] were present in the 162 upregulated genes but not in the pathway maps, which reveals a limitation of our approach. However, these five classic RF were present in seven of the ten biological processes at the same time and some of them in the other three biological processes; therefore no biological process in particular contained the known anti-HIV RFs and would have permitted us to focus on a distinct biological process. Let us remind that the major IFN-α mediated inhibition, we observed, occurs somewhere between pre-integration complex formation and proviral integration, and that the anti-HIV active genes mentioned above (i.e APOBEC3G, SAMHD1, Tetherin/Bst-2, TRIM5α) interfere with HIV-1 replication at other steps in the HIV-1 replication cycle as known so far, except for MX2, which acts between pre-integration complex formation and proviral integration[108, 124]. For example, OAS1, OAS2 and OAS3

act prior to the reverse transcription[110], PKR (EIF2AK2), during translation; ISG15 and USP18 during budding[111, 112, 114]. Therefore, these factors might not be the ones responsible for the restriction observed in MT4R5 and SupT1R5 or they act by a yet unknown mechanism(s). The following factors, IFI6[117], IRF7[116], ISG54 (IFIT2)[118], MDA-5 (IFIH1)[117], NMI[121], and RIG-I (DDX58)[119], are antiviral active but no reports about their potential anti-HIV activity exist – thus, they should be explored for their anti-HIV activity. MX1 on the other hand has been tested against HIV-1 but it does not affect its viral replication[108].

Furthermore, we compared the gene expression pattern from MT4R5 and SupT1R5 cells with the one from MT2 and MT4 cells (parental cell line) treated with IFN-α presented in the literature[125]. The 304 upregulated genes in MT4R5 cells covered the 262 genes upregulated in the MT4 cells. Even though we used a one log lower dose of IFN-α (100U/mL), we observed higher fold-changes of the individual genes for MT4R5 cells and more genes than for MT4 cells. This is easily explained by the different techniques applied: we used NGS while in the previous study, they used microarrays. NGS grants a higher coverage and higher sensitivity than microarrays[126]. The gene expression pattern of SupT1 cells (parental cell line) upon IFN-α treatment has not been reported in the literature so far.

We also compared the gene expression pattern from MT4R5 and SupT1R5 cells with the one from primary monocytes treated with IFN-α and with that of HIV-1 infection of primary tissue. Since HIV-1 infection induces a prominent IFN-α response[127, 128], this *in silico* analysis might give some hints whether the genes identified in IFN-treated MT4R5 and SupT1R5 cells might have a role in HIV-1 pathogenesis. Of note, activation of the IFN-system in HIV-1 may be a double-edged sword, on the one hand it may block HIV-1 infection[129], on the other hand, it may sustain immune activation, increasing HIV-1 pathogenesis[130]. Of the 162 genes we found, 44 were also reported in the literature in the context of IFN-α treatment or HIV-1 infection. Since it does not seem to be a difference between the genes upregulated in *in vivo* (chronic or acute) or *ex vivo* HIV-1 infection, the genes upregulated in SupT1R5 and MT4R5 cells do not seem to be particularly involved in HIV-1 pathogenesis. Of note, it was shown previously that there is no difference between the genes upregulated in CD4+ T cells from patients in acute or in chronic phase[128]. The large difference of genes upregulated within the same context might be explained by the different

cell types and the dose of IFN-α used. According to a recent publication, IFN-α induces the upregulation of antiviral genes at weak concentrations (robust genes) and at higher concentrations it upregulates other genes (tunable genes), involved in cell proliferation, chemokine activity, inflammation[131].

Interestingly, the same genes enriched in the pathway maps (see above) with anti-HIV and antiviral activity are present among the 44 genes, upregulated in our cell lines, as well as in the literature, the only exception being IRF7 and MX1. On the other hand, 11 gens with anti-HIV activity (PLSCR1[132-134], MX2[122, 123], IFITM1, IFITM2, IFITM3[135, 136], BST2[137], TRIM22[138], HERC5[139], IFI16[140], ISG20[141], RSAD2[142], LGALS3BP[143], and UBE2L6[144]) and 7 genes with antiviral activity (apart from HIV-1)(IFIT1[145], GBP1[146-148], IFI27[149], ZBP1[150], OASL[151], IFI35[152] and IFIT3[133, 153]) were present in the 44 genes upregulated in our cell lines and in the literature but not in the pathway maps, indicating again a limitation of the gene ontology approach.

Again, these genes with reported anti-HIV activities act at another step in the viral life cycle as at the one we observed (see results section 3.1). Only MX2 and IFI16 act at the same step as we observed. However, silencing MX2 by shRNA did not affect HIV-1 replication in MT4R5 and SupT1R5 cells, suggesting that another cellular factor restricts HIV-1 replication in these cell lines. The lack of any anti-HIV activity of MX2 was surprising – we speculated that either MX2 has no inhibitory HIV-1 activity in MT4R5 and SupT1R5 cells although it is highly upregulated or that a co-factor is needed for MX2 to exert its anti-HIV activity. IFI16, which acts as a DNA sensor and activates caspase, leading to apoptosis, acts between reverse transcription and integration[154] and therefore might be the RF in MT4R5 and SupT1R5 cells. However, we did not observe any apoptotic effect when MT4R5 or SupT1R5 cells were treated with IFN-α and subsequently infected with HIV-1. Nonetheless, we believe exploring IFN's effect on HIV-1 should be explored when IFI16 silenced in our cell lines prior to explore other potential RF.

Strength on the one hand, but also a limiting factor on the other hand is that the initial 162 genes identified in IFN-treated MT4R5 and SupT1R5 cells were selected based on their 2 fold upregulation and using the gene ontology approach. The synopsis of all the gene expression

studies done in various systems points to a rather "small" set of genes upregulated, which must be explored in depth for their anti-HIV activity. Although the gene ontology approach provides a valuable access to the function and partners of the different genes upregulated, it does not necessary provide a list with the definitive anti-HIV candidates. In particular, since we focused mainly on the pathway maps, several other genes identified by biological processes or the process networks could be excluded. If the genes identified using the pathway maps do not correspond to an anti-HIV RF, data should be analysed differently (e.g. using the biological processes), although it will be much more difficult to interpret due to the large number of genes enriched.

Outlook*:* Since RIG-I (DDX58) is a gene responsible for the induction of IFN-α/β[119] and therefore is involved in a 2nd wave of gene upregulation[155], we could in a first step silence RIG-I (DDX58) and determine if the anti-HIV effect of IFN-α is abolished in MT4R5 and SupT1R5 cells. This could point to a particular transduction pathway and exclude all the effector proteins upregulated in the 1st wave of gene upregulation. If the silencing of RIG-I (DDX58) does not counteract the antiviral effects of IFN-α, there is a second approach: The genes with antiviral activity enriched in the pathway maps could be tested, to determine their anti-HIV effects by overexpressing them in MT4R5 and SupT1 and depending on the data, by their silencing. Their overexpression and subsequent suppression of HIV-1 replication in MT4R5 and SupT1R5 cells would indicate that they are the RF that we are looking for and their silencing and subsequent loss of IFN-α anti-HIV effect would confirm that they are the responsible RF. Such RF would need to be tested in other cell lines and more important, in primary cells, to determine if its effect is relevant in the target cells of HIV-1, first *in vitro* and if the results are promising, *in vivo*. However, the gene ontology analysis has its limitations, for instance the classical RF did not show up in the pathway maps analysis. Therefore it would be risky to focus only on such a limited number of candidate genes. It would make more sense to perform a blind screening with shRNAs targeting all 162 genes upregulated in MT4R5 and SupT1R5 cells, however this approach is very expensive and cumbersome.

Since we identified the step at which viral inhibition takes place, i.e. between reverse transcription and integration, an alternative approach would be the immuno-precipitation of the pre-integration complex, with or without IFN-α and a subsequent identification of the proteins present only in the IFN-α treated cells, using mass spectrometry. The proteins bound

to the pre-integration complex only when the cells are treated are likely to be a RF. Of note, a similar approach was already taken to identify the host proteins in infected CD4+ cells[156], but not after IFN-α treatment, like we suggest.

4.2 The development of humanized mice models to study mechanisms of HIV infection

4.2.1 Long-acting anti-retroviral drugs for treating HIV-1 in humanized mice

The second part of my thesis work focused on studying the impact of the RF APOBEC3G (A3G) *in vivo* in humanized mice, in particular the emergence of mutations linked to it. My first experience with humanized mice as a small animal model for studying HIV infection *in vivo* came from work done with my peer Marc Nischang. We showed that mice treated with cART including a novel long-acting (LA) non-nucleoside-analogue, TMC278-LA had undetectable viral load. Similar as in human, after suppression of viral load with cART and subsequent interruption of cART or replacement of cART with only TMC278-LA (monotherapy) led to a rapid rebound of the viral load. Thus, TMC278-LA is not potent enough as mono-therapy to supress HIV RNA. Furthermore, the viral rebound clearly points to a latent reservoir of HIV-1. The mice with viral failure showed emergence of resistance mutations, i.e., the key signature mutations M184I and E138K of the nucleoside reverse transcriptase inhibitor (NRTI) 3TC and of the non-NRTI (NNRTI), TMC278-LA resp. TMC278-LA in combination with a protease inhibitor, TMC181_LA, both in a nano-based long-acting formulation was so. As expected, this treatment also preserved the CD4+ T-cell count as quantified by the CD4/CD8 ratio, and it even resulted in recovery of the CD4+ T-cells in those mice with a previous CD4+ T-cell decline. Thus, long-acting compounds which are given on a weekly or monthly basis might be a realistic treatment option in particular for HIV-infected patients with poor drug adherence. *Ex vivo* we provided convincing evidence that HIV establishes a latent reservoir by detecting proviral DNA in splenocytes obtained from HIV-infected mice with undetectable HIV DNA. Using a cocktail of compounds, this latent reservoir was reactivated as measured by the expression of HIV-1 Gag transcripts.

To summarize this work convincingly proves that humanized mice represent a valuable small mouse model for studying HIV pathogenesis and novel treatment approaches. The latent reservoir represents the major hurdle for cure of HIV. This mouse model will be very useful for studying novel treatment approaches for the eradication of this reservoir, i.e., we are now able to test different compounds for its potential to reactivate viral transcription in a model that approximates the human situation - compounds will be tested such as deacetylation inhibitors, methylation inhibitors or chromatin-modifying agents. The contribution I made in this work is reflected by a co-authorship on the publication.

4.2.2 High resolution mapping of HIV drug resistance evolution driven by APOBEC3G

I also worked on my own project using the humanized mouse, i.e. we studied the biological properties of the Vif-APOBEC3 axis, in particular the spectrum of APOBEC3 proteins, which hypermutate the HIV-1 genome, which are in turn neutralized by the HIV-1 protein Vif. We hypothesized that partial neutralization of APOBEC3-editing enzymes (as illustrated by the 45G mutant) induces mutations that decrease viral fitness, but under certain conditions, such as under the pressure of the immune system or drug treatment, might be beneficial for viral escape. APOBEC3-editing enzymes counteract HIV-1 and other retroviruses by hypermutating their viral genome[65, 66]. APOBEC3G and APOBEC3F are neutralized by the accessory protein Vif present in wild-type HIV-1[67, 157]. The significance of these enzymes for HIV-1 infection is demonstrated by the lack of replication when challenging mice with HIV-1 strains deficient in Vif. However, partially active Vif might lead to a partial neutralization of the APOBEC3-editing enzymes, which might favor viral diversification and thus contributing to viral escape from the immune system or drug treatment.

We looked at the mutagenesis induced by the cellular APOBEC3 deaminases in the context of viruses that harbor point mutations in the Vif protein. Vif has a region which binds to the different APOBEC3-editing enzymes[67, 68] and another region which binds to the proteasomal machinery, in order to send the bound APOBEC3-editing enzymes for their degradation[157, 158]. The 45G mutant is not able to bind and therefore degrade APOBEC3G but the other APOBEC3-editing enzymes[68]. The SLQ mutant has a mutation in the SLQ domain, which interferes with the binding of the proteasomal machinery and therefore is

unable to induce the degradation of the different APOBEC3 proteins and thus has been considered as an equivalent of a Vif-deficient virus [158-160]. This work was done in close collaboration with Dr. Viviana Simon (Mount Sinai School of Medicine, New York City). Dr. Simon and co-workers using a rather artificial system demonstrated the development of drug resistance when there is mixture of Vif mutants and Vif WT population of viruses[161]. In such experiments three conditions were tested *in vitro* at high concentrations of 3TC: HIV-1 WT replication, HIV-1 Vif mutant 45G replication (cannot counteract APOBEC3G) and a combination of both. Only the combination of both viruses was able to replicate under these conditions, as opposed to the single viruses, indicating that the 45G mutant is able to transfer the drug resistance mutation to the WT virus, possibly by recombination[161].

Based on previous experiments *in vitro*, we wanted to show that 45G mutant virus is able to develop drug resistance faster than the WT version, *in vivo*, and therefore we performed the experiments in the humanized mouse. For this, we infected mice with either HIV-1 WT or Vif mutants 45G or SLQ (cannot counteract any APOBEC3).

In vitro, using either PBMCs or cord blood cells depleted from the CD34+ hematopoietic precursor cells, we observed that the WT and the 45G viruses replicate at the same level, as observed by our collaborator[161]. In contrast, we observed surprising results *in vivo*: the WT virus and the 45G mutant replicated similarly during the first 4 weeks, but after 8-12 weeks, the replication of the 45G mutant decreased significantly which was not the case for the WT virus. Thus, the viral fitness of the 45G mutant is indeed lower than the one of the WT but this is only recognized after a certain lag time. The SLQ mutant HIV-1 strain, unable to degrade any APOBEC3 protein, did not replicate at all, showing the importance of the neutralization of the APOBEC3 family members for successful HIV-1 replication *in vivo*.

To validate the hypothesis that selective pressure (e.g. drug treatment) might be beneficial for the viral escape of the 45G as compared to the WT virus, we infected humanized mice with either WT or 45G viruses and 4 weeks later started treatment with 3TC (food pellets) to determine if the 45G mutant develops drug resistance faster than the WT virus, as shown *in vitro*[161]. We monitored the viral load every two weeks and performed ultra-deep sequencing on the viral RNA from plasma in order to determine viral evolution overtime. While the initial decline in HIV-1 RNA was similar with both HIV-1 strains, the viral

rebound was significantly faster in the 45G-infected mice than in WT-infected mice. One could expect that the faster rebound observed in 45G-infected mice is due to a faster development of 3TC resistance mutations - however the NGS data showed that this is not the case: irrespective of wild-type or 45G strains, NGS data showed 3TC resistance in almost all mice after two weeks. A closer look revealed that the quasi-species in mice infected with the 45G virus had mainly M184I mutations, which correspond to G-to-A mutations, typical of APOBEC3G, while the quasi-species in mice infected with the WT virus presented both mutations M184I and M184V, typical of stochastic mutations. The mutation M184I increases the RT fidelity 4-fold and M184V 2.5-fold as compared to WT RT[162, 163]. We speculate that the higher fidelity of the RT with M184I may prevent an excessive RT-driven diversification and thus, HIV-1 strains with M184I might have a replication advantage as compared to HIV-1 strains with M184V or WT RT. In other terms, the gain of RT fidelity may outweigh the APOBEC3G editing effects. However, the gain of viral fitness of the 45G mutant is only apparent under drug or immune pressure promoting specific mutation as illustrated here with 3TC since viral replication was attenuated overtime without 3TC treatment.

In conclusion, a partial neutralization of APOBEC3 family members might be beneficial for HIV-1, not for a faster development of drug resistance mutations, as initially thought, but to increase viral fitness under selective pressure. In this case, the selective pressure was the 3TC drug, but this could also be extrapolated to other drugs or the selective pressure by the adaptive immune system.

This work showed that APOBEC3G partial neutralization decreases viral fitness of HIV-1 but under selective pressure it increases viral fitness. And since HIV-1 is present as quasi-species population and has a high rate of mutation, mutations of Vif, rendering it partially active might happen in some strains, as shown in patients[68], APOBEC3G could actually favor HIV-1 drug resistance or immune escape.

Additionally, this works illustrates very convincingly the power of the Illumina platform for next-generation sequencing of HIV-1 quasi-species from the plasma and in particular for detecting resistance mutations. Before 2007, previous sequencing studies in the field of HIV-1 used rather exclusively the Sanger's sequencing-based PCR amplification, which permitted to

identify the sequence of the most prevalent HIV-1 strain but did not allow to discriminate quasi-species or to detect minor HIV-1 populations[164, 165].

So far, in the HIV field, the Illumina platform has only been used a few times *in vitro*[166-168]. The advantage of this method is that it is appropriate for divergent viruses, or for samples with low amounts of viral RNA, like in the case of patients under cART, allowing to detect most or all the different quasi-species populations[169]. Our study underlines the value of the Illumina NGS platform to detect HIV-1 resistance in plasma viral RNA with high accuracy.

Outlook: It was discussed that the conclusions obtained in the experiments showing the increase in viral fitness of the mutant 45G under selective pressure (3TC treatment) require confirmation and further exploration due to the astonishing results. Therefore, new experiments have been set and have been ongoing in the last months, therefore the submission of the manuscript has been postponed.

4.2.3 Gene therapy against HIV-1 using the humanized mice

I was also involved in studying gene therapy approaches to render the immune system resistant to HIV-1 (manuscript published). My peer, Renier Myburgh had the lead in this work. Here, we transduced CD34+ hematopoietic stem and precursor cells (HSPCs) with a lentiviral vector containing miRNAs against the HIV co-receptor CCR5. CCR5 together with CD4 constitutes the HIV receptor complex – absence of CCR5 renders cells non-permissive to HIV. We observed a substantial decrease in the HIV load in the mice transplanted with HSPCs gene engineered with the above-mentioned lentiviral construct as compared to the control mice (functional cure); in parallel, we also observed a preservation of CD4+ T cells and a homeostatic expansion of memory CD4+ T-cells. This work clearly showed that gene therapy strategies are a promising approach to cure HIV-1.

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